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MECHANISMS OF NOTCH-MEDIATED INHIBITION OF SKELETAL MYOGENESIS

Abstract
The Notch pathway is an evolutionarily conserved signaling cascade that regulates many cell fate decisions. Recent work has revealed that Notch plays critical roles in the control of skeletal muscle development and regeneration. In the embryo, Notch maintains a pool of myogenic progenitor cells and prevents their premature differentiation. In the adult, after muscle injury, Notch signaling is essential for the initial expansion of muscle stem cells, or satellite cells.

While it has been known for over a decade that Notch activity represses myogenic differentiation, the molecular mechanisms by which this inhibition occurs are poorly defined. In this thesis, I sought to identify the key transcriptional effectors of Notch in muscle and explore how these proteins repress the myogenic program. Using the mouse myoblast cell line C2C12, I identified 82 transcripts upregulated after six hours of ligand-mediated Notch stimulation. When constitutively expressed in myoblasts, several of these genes (Nrarp, HeyL, Trib2) had no apparent impact on differentiation, while at least two of them, the canonical effector Hey1 and the novel Notch-responsive gene MyoR, were capable of recapitulating the pathway's inhibitory effects. Interestingly, siRNA knockdown of Hey1 or MyoR, or the two factors in combination, failed to rescue the differentiation of myoblasts exposed to Notch ligands. These results support a model in which Notch acts through multiple, potentially redundant pathways to repress myogenesis.

In subsequent work, I focused on the mechanistic question of how the Notch effector Hey1 interferes with myogenic transcription. My functional and biochemical data revealed that Hey1 does not target the inherent transcriptional activity of the skeletal muscle master regulator MyoD. I found that Hey1 repressed only a subset of MyoD target genes, and consistently, did not disrupt dimerization of MyoD with its obligate binding partner E47. My results indicated that Hey1 is recruited to the promoter regions of Myogenin and Mef2C, two genes whose induction is critical for differentiation. Expression of Hey1 in C2C12 myoblasts correlated with reduced recruitment of MyoD to these promoters, arguing that Hey1 inhibits myogenesis by associating with and repressing expression of key myogenic targets.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Cell & Molecular Biology

First Advisor
Tom Kadesch

Keywords
Notch, myogenesis, skeletal muscle, satellite cells

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Subject Categories
Cell and Developmental Biology | Molecular Genetics

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MECHANISMS OF NOTCH-MEDIATED INHIBITION OF SKELETAL MYOGENESIS

Matthew F. Buas

A DISSERTATION

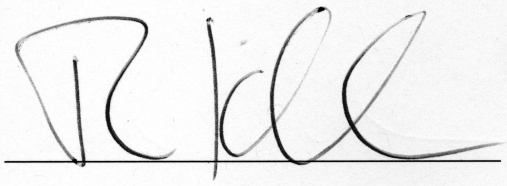
in

Cell and Molecular Biology

Presented to the Faculties of the University of Pennsylvania

in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

2009



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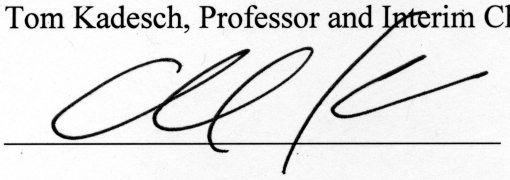

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ACKNOWLEDGEMENTS

I first and foremost would like to acknowledge my thesis advisor, Tom Kadesch, for his generous mentorship and support throughout my Ph.D. journey. I am grateful to Tom for his critical scientific eye and unwavering optimism, and for our many interesting discussions about science that I will remember for years to come. I would also like to thank Kadesch lab members past and present. Shara Kabak, a former postdoc, and Ruth McCarrick-Walmsley, our former lab manager, were instrumental in smoothing my transition to the lab—they welcomed me aboard and provided patient assistance in working through technical obstacles. Yao-juan Liu, our technician, has been an enormous help in day-to-day lab operations, and her general good cheer has been much appreciated. I would also like to thank members of my thesis committee for their advice and input—Jon Epstein, Beth Barton, Steve DiNardo, and Warren Pear. I acknowledge John Tobias for his help with microarray analysis, and Doug Epstein and Diane Dolson for providing us with in-situ hybridization data. Last but not least, I thank my parents for their encouragement.

ABSTRACT

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Matthew F. Buas

Tom Kadesch, Ph.D.

The Notch pathway is an evolutionarily conserved signaling cascade that regulates many cell fate decisions. Recent work has revealed that Notch plays critical roles in the control of skeletal muscle development and regeneration. In the embryo, Notch maintains a pool of myogenic progenitor cells and prevents their premature differentiation. In the adult, after muscle injury, Notch signaling is essential for the initial expansion of muscle stem cells, or satellite cells.

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knockdown of Hey1 or MyoR, or the two factors in combination, failed to rescue the differentiation of myoblasts exposed to Notch ligands. These results support a model in which Notch acts through multiple, potentially redundant pathways to repress myogenesis.

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Chapter I. Introduction

Part 1: Skeletal myogenesis

The generation of skeletal muscle tissue is a highly regulated, step-wise process that is controlled in vertebrates by a family of four homologous transcription factors, known as muscle regulatory factors (MRFs). The founding member of this family, MyoD, was discovered over twenty years ago and shown to exhibit the remarkable ability to convert a wide range of non-muscle cell lines into the muscle lineage (Davis et al., 1987; Weintraub et al., 1989). Since the initial cloning and characterization of this master regulator, skeletal muscle has become one of the best studied systems of cellular differentiation. The availability of cell culture models, which recapitulate many aspects of in vivo myogenesis, has greatly facilitated the molecular and biochemical dissection of the myogenic transcriptional program.

Muscle regulatory factors

MyoD was discovered by Weintraub and colleagues, who first made the intriguing observation that rare colonies of 10T1/2 fibroblasts transfected with DNA from 5'azacytidine-treated fibroblasts were converted to a muscle phenotype (Lassar et al., 1986). This finding was interpreted to suggest that a structural change, likely demethylation, of a single, normally-silent DNA locus within fibroblasts was capable of inducing myogenesis. Subtractive cDNA hybridization was then employed to identify a cDNA, MyoD, that exhibited this potential and shared sequence homology to the transcription factors myc and achaete-scute (Davis et al., 1987). Consistent with a role as

skeletal muscle master regulator, MyoD was found to be expressed exclusively in this lineage in vivo, and only in myogenic cell lines in vitro. Soon after the identification of MyoD, three other homologous muscle-specific transcription factors—Myf-5, Myogenin, and MRF4—were also cloned and shown capable of converting cultured fibroblasts to a muscle phenotype (Braun et al., 1990; Braun et al., 1989; Wright et al., 1989).

MyoD and the other MRFs belong to a family of transcription factors known as basic helix-loop-helix (bHLH) proteins (Figure 1.1). These factors bind to E-box elements (CANNTG) within DNA to activate transcription. MyoD requires both its basic domain, for DNA binding, and its HLH motif, for dimerization with other bHLH factors known as E-proteins, such as E2A, E2-2, and HEB. While MyoD homodimers may assemble and function with low efficiency, an important role for heterodimerization was suggested by several lines of evidence—MyoD-E protein heterodimers are the predominant species in myoblast nuclear extracts, E proteins synergize with MyoD in transcriptional reporter assays, and anti-sense E47 impairs MyoD-mediated conversion of fibroblasts (Lassar et al., 1991).

One of the early puzzles in transcriptional control by MyoD was the fact that its DNA recognition element, the E-box, was found not only in myogenic target gene promoters, but also in many promoters not activated in muscle. Conversely, non-myogenic bHLH proteins, such as ubiquitously expressed E proteins, were found to bind to myogenic E-boxes but failed to activate these promoters. In vitro DNA binding capacity alone did not account for transcriptional activity. The basis for specificity was first explored by Weintraub and coworkers, who demonstrated that two residues present within the MyoD basic domain but absent from other bHLH proteins, an alanine and

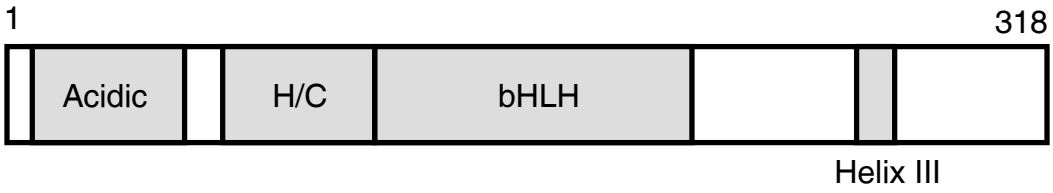

Figure 1.1. Structure of the MyoD protein. The N-terminal acidic domain functions in transcriptional activation, the H/C and Helix III motifs participate in chromatin remodeling, and the bHLH (basic helix-loop-helix) domain mediates DNA binding and dimerization. Adapted from Berkes et al. (2004).

threonine, were critical for transcriptional activity on myogenic promoters (Davis and Weintraub, 1992; Weintraub et al., 1991). It was proposed, and later demonstrated, that these residues are important for collaboration of MyoD with critical cofactors, namely members of the Mef2 family of transcription factors (Molkentin et al., 1995). In parallel, other work suggested that the inability of MyoD to activate E-box-containing non-muscle promoters, such as the immunoglobulin heavy chain (IgH) enhancer, was in part due to the presence of negatively acting cis-elements. It was postulated that nucleotides flanking the E-box could potentially recruit a repressor acting specifically on MyoD but not E47 (Weintraub et al., 1994).

Skeletal muscle development

During mammalian embryogenesis, skeletal muscle tissue of the body and limbs is derived from the somites, regularly patterned blocks of paraxial mesoderm adjacent to the neural tube. Somites are subdivided into a ventral compartment, the sclerotome, and a dorsal compartment, the dermomyotome. While the sclerotome gives rise to ribs, vertebrae, and discs, the dermomyotome gives rise to skin and skeletal muscle. The dermomyotome can be further divided into a medial portion, from which epaxial muscles of the back and intercostals are derived, and a lateral portion, from which hypaxial muscles of the ventral body wall and limbs are derived. Myogenesis is a step-wise process whereby proliferating myogenic progenitors give rise to a population of myoblasts, which continue to divide before exiting the cell cycle, differentiating, and fusing into multinucleated myotubes (Figure 1.2). Bundles of parallel myotubes then form a mature myofiber, the basic contractile unit of skeletal muscle. The myotome, a

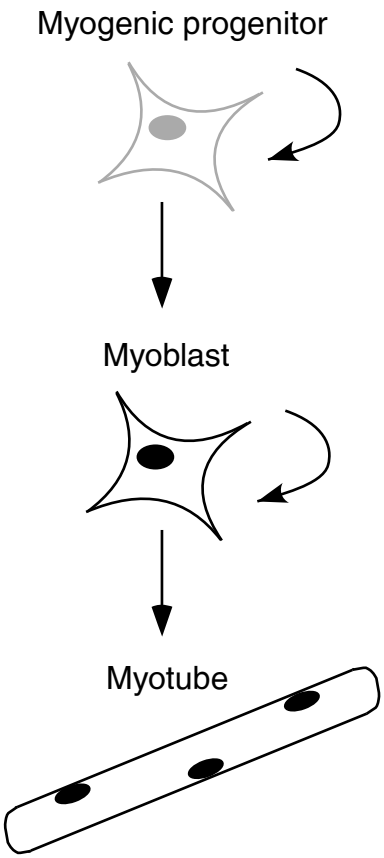

Figure 1.2. Skeletal myogenesis. Myogenic progenitors proliferate and give rise to a population of myoblasts, which continue to divide before exiting the cell cycle, differentiating, and fusing to form multinucleated myotubes.

sheet of muscle precursors between the sclerotome and dermomyotome, represents the first skeletal muscle mass to be formed. Primary myofibers arise from the fusion of embryonic myoblasts and are thought to serve as a framework on which myoblasts continue to proliferate before fusing into secondary myofibers during fetal development (Kelly and Zacks, 1969).

Gene knockout studies in the 1990s established critical roles for the MRFs in controlling skeletal myogenesis in the embryo. While muscle development proceeded normally in MyoD or Myf-5 single knockout animals, MyoD/Myf-5 double knockouts died soon after birth with virtually a complete absence of skeletal muscle (Braun et al., 1992; Rudnicki et al., 1992; Rudnicki et al., 1993). This initial finding implied that MyoD and Myf-5 can compensate for each other in myogenic specification. In stark contrast to the single knockouts above, deletion of Myogenin resulted in a severe reduction of skeletal muscle, and an apparent block in the ability of myoblasts to terminally differentiate (Hasty et al., 1993; Nabeshima et al., 1993). The MRF4 knockout was viable and fertile, and a significant upregulation of Myogenin was observed, suggesting potential compensation by this pro-differentiation factor (Zhang et al., 1995). Later studies suggested that MRF4 may also contribute to specification in parallel with MyoD and Myf-5 (Kassar-Duchossoy et al., 2004). The picture that emerged from these genetic studies was a hierarchical relationship among the MRFs, in which MyoD and Myf-5 (and likely MRF4) were involved in the initial specification of myogenic precursors, while Myogenin functioned downstream in promoting terminal differentiation.

One interesting question raised by the MRF knockout studies was whether the divergent phenotypes observed were a consequence of inherent functional differences among these four proteins, or were merely a reflection of differences in expression patterns. For example, it was possible that Myogenin (or MRF4) was unable to rescue myogenic specification in the MyoD/Myf-5 double knockout simply because its expression in embryogenesis is delayed relative to Myf-5 (Ott et al., 1991; Sassoon et al., 1989). To address this possibility genetically, the Myogenin coding region was knocked in to the Myf-5 locus, and resulting mice were crossed to the MyoD knockout. Importantly, in this background, expression of Myogenin in the same spatiotemporal pattern as Myf-5 only partially rescued myogenesis and was not sufficient to prevent perinatal lethality resulting from compromised muscle formation (Wang and Jaenisch, 1997). This result indicated that Myogenin was not as intrinsically capable as Myf-5 in the process of specifying or maintaining muscle precursors. Consistent with the idea of functional divergence, later studies by Tapscott and colleagues in cultured fibroblasts further revealed that MyoD and Myf-5 were markedly more efficient than Myogenin in remodeling chromatin and inducing transcription at endogenous muscle promoters (Gerber et al., 1997). By contrast, the MRFs were equivalent in their ability to activate transfected reporters driven by E-boxes. These results suggested that the capacity of MyoD and Myf-5 to function efficiently in myogenic specification might derive from an ability to activate genes in silent chromatin. Mapping experiments indicated that the remodeling activity of MyoD could be localized to a histidine/cysteine (H/C)-rich N-terminal domain and a C-terminal alpha-helix (Helix III), conserved in the Myf-5 but not Myogenin coding sequence (Figure 1.1). Domain swapping confirmed that these motifs

within MyoD could render Myogenin capable of efficiently inducing silent endogenous genes (Bergstrom and Tapscott, 2001). Interestingly, instead of encoding chromatin remodeling activity, the Myogenin C-terminus was shown to contain a general transcriptional activation domain, further providing a molecular explanation for the distinct activity exhibited by this downstream pro-differentiation MRF.

Other work revealed the upstream signals responsible for initiating MRF expression within the developing somite. Explant studies showed that the inducing activities of axial structures such as the floor plate and dorsal neural tube could be mimicked by the signaling molecules sonic hedgehog and Wnt1, 3, 4, respectively (Munsterberg et al., 1995). Wnts and Shh were further shown to act upstream of two paired-box family transcription factors, Pax3 and Pax7. Forced retroviral expression of Pax3 in chick somite explants was capable of activating expression of Myf-5 and MyoD in the absence of axial tissues (Maroto et al., 1997). Conversely, mice homozygous null for both Pax3 and Myf-5 lacked body muscles and MyoD expression, suggesting that Pax3 and Myf-5 lie genetically upstream of MyoD activation; consistently, Myf-5 RNA is induced approximately two days prior to MyoD during development (Tajbakhsh et al., 1997). Interestingly, Pax3 single knockout animals lacked limb muscles (Bober et al., 1994; Goulding et al., 1994), likely a consequence of impaired induction of c-met, a receptor critical for the proper migration of muscle precursors from the somite to the limb bud (Epstein et al., 1996; Yang et al., 1996). Within the somite, the domain of Pax3 activity is further restricted to the dorsal medial lip (DML) of the dermomyotome by signaling through bone morphogenic proteins (BMPs). Specifically, BMPs inhibit

MyoD/Myf5 activation in $Pax3^{+}$ cells, except in the DML, where the BMP antagonist noggin relieves this repression and allows for MRF expression (Reshef et al., 1998).

Post-natal myogenesis: satellite cells

Skeletal muscle has long been known to possess the ability to regenerate in response to injury (Carlson, 1973). This regenerative capacity has been ascribed to the presence of a normally quiescent population of mononuclear cells known as satellite cells (SCs), situated between the basal lamina and the muscle fiber membrane (Mauro, 1961). In response to tissue damage, SCs exit quiescence, become activated, proliferate, and either differentiate and fuse to form new myofibers or return to their ground state and repopulate the SC niche (Figure 1.3). A number of independent studies provided support for the notion that satellite cells possess the hallmark properties of stem cells—the ability to self-renew and differentiate to maintain tissue homeostasis (Collins et al., 2005; Montarras et al., 2005; Sacco et al., 2008). An emerging theme from this work is that despite the original definition of a satellite cell based on anatomic location, all satellite cells are not equivalent, and only a subset may exhibit true stem-cell properties.

Various markers have been used to identify satellite cells prospectively, but one of the first and most commonly employed is the transcription factor Pax7. Initial studies demonstrated that mice homozygous null for Pax7 exhibit grossly normal muscle, but completely lack satellite cells, fail to thrive, and die after two weeks (Seale et al., 2000). The defect was originally postulated to be at the level of SC specification, but later work argued instead that Pax7 is required for SC maintenance and survival, potentially acting to inhibit SC apoptosis (Oustanina et al., 2004; Relaix et al., 2006). It now appears that

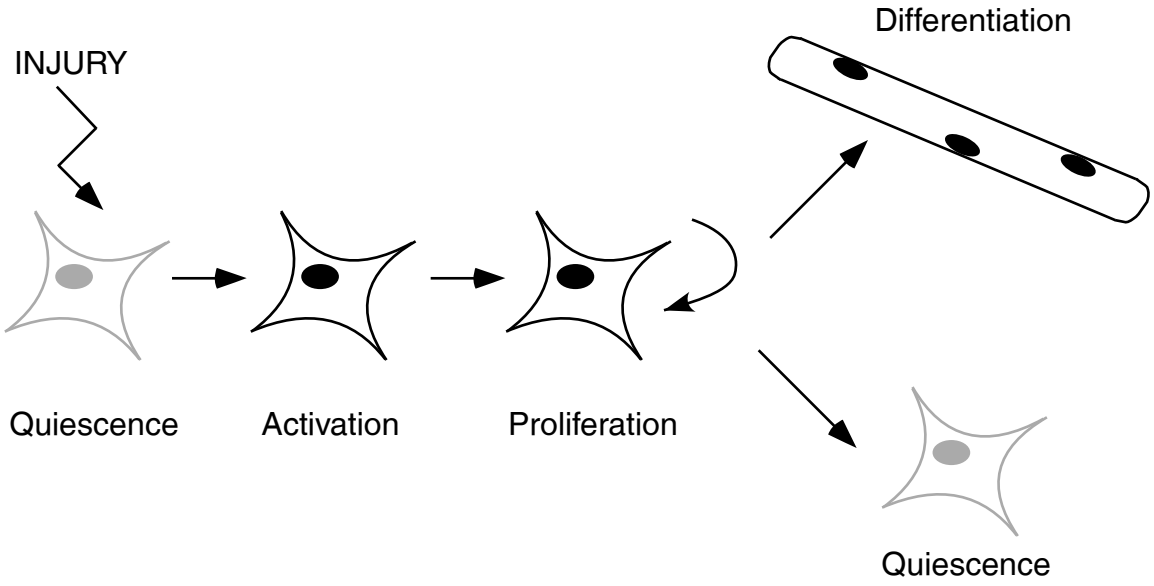

Figure 1.3. Satellite cell-mediated muscle regeneration. Upon injury, satellite cells exit quiescence, become activated, proliferate, and either differentiate and fuse to form multinucleated myotubes or return to quiescence.

Pax7 is necessary for satellite cell survival and function only during the first few weeks of postnatal life, when SCs are still making the transition to their ultimate state of quiescence (Lepper et al., 2009). Recent studies have established that satellite cells and embryonic muscle progenitors share a common embryological origin, a proliferating pool of $Pax3^{+}/Pax7^{+}$ cells that arise within the central dermomyotome of the somite (Gros et al., 2005; Kassar-Duchossoy et al., 2005; Relaix et al., 2005). Pax7 appears to be uniformly retained in SCs after birth, whereas Pax3 expression is only detected in subsets of SCs in certain muscles, and apparently cannot compensate for Pax7's functions in SCs maintenance (Relaix et al., 2006).

While satellite cells have long been considered a resident stem cell pool and the primary source of muscle regenerative potential, conclusive evidence for this notion was obtained only in the last five years. Multiple groups have prospectively isolated satellite cells from mice and demonstrated their ability to engraft into the endogenous SC compartment of injured mice and contribute to repair (Cerletti et al., 2008; Montarras et al., 2005). Single myofibers with their associated satellite cells have also been transplanted into injured muscles, and SC contributions to regeneration have been verified (Collins et al., 2005). In such studies, donor SCs are often isolated from transgenic animals such that they are marked, for example with GFP, and can easily be tracked in vivo upon engraftment into host animals. Importantly, such work has shown that engrafted satellite cells not only contribute to formation of regenerating muscle, but also reseed the SC niche and can participate in multiple rounds of repair after repeated injury (Cerletti et al., 2008; Collins et al., 2005; Sacco et al., 2008). These findings argue strongly that SCs exhibit self-renewal and the ability to maintain an adult tissue.

Interestingly, other cellular reservoirs—bone marrow-derived cells, "side population" muscle cells, and $CD45^{+}Sca1^{+}$ resident myogenic cells—have also been proposed to contribute to the muscle repair process (Ferrari et al., 1998; Gussoni et al., 1999; Polesskaya et al., 2003); these populations, however, do not exhibit the equivalent properties of satellite cells and are now believed to play only a negligible role in regeneration (Sherwood et al., 2004).

Studies on cultured myofibers and associated satellite cells in vitro in combination with in vivo injury experiments have helped define the sequence of molecular events accompanying SC activation. In the quiescent state, SCs are $Pax7^{+}$ but devoid of MRF expression; upon activation, they first upregulate MyoD and/or Myf-5 (Cooper et al., 1999; Cornelison and Wold, 1997). While most of these cells continue to proliferate, downregulate Pax7, and ultimately express Myogenin/MRF4 upon entry into the differentiation pathway, some retain Pax7 and lose MRF expression (Cornelison and Wold, 1997; Zammit et al., 2004; Zammit et al., 2006). It is these $Pax7^{+}/MRF^{-}$ cells that exit the cell cycle and return to a state of quiescence to replenish the stem cell niche. Interestingly, as in the embryo, MyoD/Myf-5 and Myogenin/MRF4 appear to play divergent roles, with the first two proteins apparently functioning in the activation/specification phase of satellite cell-mediated regeneration, and the latter two factors operating downstream in promoting differentiation. In contrast to the largely redundant functions of MyoD and Myf-5 during embryogenesis, however, MyoD was found to play a unique role in postnatal regeneration, as the MyoD knockout mouse displayed a significantly impaired response to muscle injury (Megeney et al., 1996). In the absence of MyoD, there appeared to be a defect in the proliferative expansion of

activated, committed satellite cells, suggesting that MyoD is required for SCs to enter the proliferative phase and progress down the lineage pathway.

A recent study provided compelling evidence that satellite cells as defined anatomically are a heterogeneous population, only a small fraction of which may exhibit true stem-cell potential (Kuang et al., 2007). By crossing the ROSA26-YFP mouse to a Myf-5-Cre line, Rudnicki and colleagues were able to permanently mark cells that at any point in their history expressed Myf-5. Strikingly, they found that ~10% of sublaminar $Pax7^+$ SCs were YFP^-. It was later demonstrated in single fiber cultures that YFP^- SCs divide asymmetrically to generate a YFP^+ daughter cell in apposition to the muscle fiber membrane, and a YFP^- daughter adjacent to the basal lamina. Upon transplantation, purified YFP^- SCs expanded rapidly and contributed both to myofiber differentiation and to the endogenous SC niche, while YFP^+ SCs failed to expand or reseed the niche. These results imply that satellite cells comprise at least two populations, one of which never expresses Myf-5, remains in continuous contact with the basal lamina during cell division, and likely represents the pool of muscle stem cells. Interestingly, follow-up studies in the past year have revealed that non-canonical Wnt signaling, initiated by Wnt7a, appears to stimulate the symmetric expansion of satellite stem cells (YFP^-) and thereby enhance the regenerative process (Le Grand et al., 2009).

The myogenic transcriptional program

Cell culture-based models of myogenesis have greatly facilitated the investigation of how MRFs initiate and drive forward a transcriptional differentiation program on a molecular level. Myogenic transcription proceeds in a strict temporal sequence from early

to late gene expression (Andres and Walsh, 1996; Bergstrom et al., 2002). This cascade is highly regulated by positively acting feedback loops and finely tuned by a variety of signaling pathways that ultimately target individual transcription factors and/or bring about key chromatin alterations.

Much work has been directed at elucidating the transcriptional control of the Myogenin locus, as this gene plays an essential role in inducing differentiation, and its regulation has illustrated important general concepts about myogenic transcription. In the early 1990s, studies in transgenic reporter mice revealed that a 133-bp proximal promoter was sufficient to recapitulate the normal temporal and spatial pattern of Myogenin expression in the developing embryo (Yee and Rigby, 1993). Two DNA binding elements, an E-box (E1) and Mef2 element, were found to be essential for the induction of Myogenin transcription (Cheng et al., 1993), while a third element, Mef3, was later shown also to be necessary (Spitz et al., 1998) (Figure 1.4). The presence of a paired E-box and Mef2 element is a common motif present within many myogenic promoters and facilitates cooperation between the MRFs and the Mef2 family of transcription factors. In mammals, there are four Mef2 factors (Mef2A, B, C, and D), all of which share a common MADS box and MEF2 domain which allow for DNA binding and dimerization (Black and Olson, 1998). MRFs and Mef2 factors physically interact and can synergize with one another (Molkentin et al., 1995). Importantly, Mef2C is itself a direct transcriptional target of MyoD (Dodou et al., 2003; Wang et al., 2001). Once expressed, Mef2C collaborates with MyoD in the activation of the Myogenin promoter (Figure 1.5) (Edmondson et al., 1992), setting up what has been proposed as a “feed forward” model underlying the myogenic program (Tapscott, 2005). This concept of MyoD collaborating

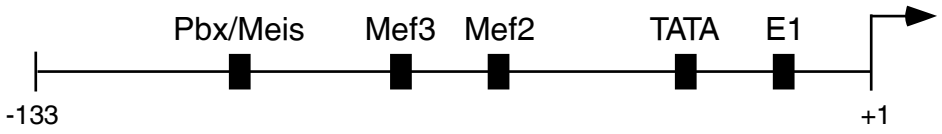

Figure 1.4. Myogenin proximal 133-bp promoter. DNA binding sites are shown for muscle regulatory factors (E-box E1), Mef2, Six1,4 (Mef3), and Pbx/Meis.

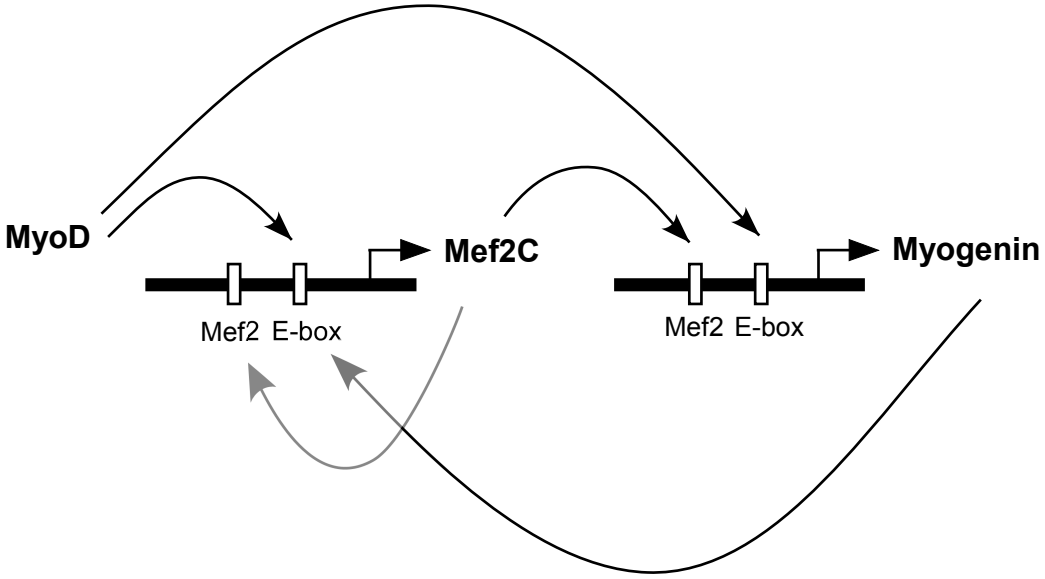

Figure 1.5. Myogenic transcriptional circuitry. The Myogenin and Mef2C promoters both contain E-box and Mef2 DNA binding elements. MyoD directly induces the expression of Mef2C, and the two factors cooperatively induce Myogenin. Positive feedback occurs as shown.

with downstream mediators to drive forward sequential gene expression represents a recurrent theme in myogenic transcription. To add another layer of control, Myogenin also feeds back to enhance expression of Mef2C, and MRFs themselves have been shown capable of inducing their own transcription (Ridgeway et al., 2000; Thayer et al., 1989). The net result is that initial, small expression changes become amplified and self-reinforced and enable a robust transcriptional output. In addition to the synergistic regulation conferred by MRFs and Mef2, members of the Six family of homeodomain proteins (Six1, Six4) have also been implicated in the control of Myogenin transcription (Spitz et al., 1998). Six factors associate with the essential Mef3 DNA binding element located distal to the Mef2 site and are thought to participate in cooperative induction.

Regulation of MyoD

A long-studied and still-relevant challenge in the field of myogenesis has been to unravel the multiple modes of regulation that control MyoD transcriptional activity. In cultured myoblasts, MyoD is expressed, yet the induction of Myogenin and downstream targets does not occur until differentiation is triggered either by removing serum or by allowing cells to reach confluence, both of which initiate cell cycle exit. This implies that the proliferative state of myoblasts may exert negative control over MyoD-mediated transcription. Consistent with this idea, forced expression of cyclin D1 inhibits MyoD activity and correlates with MyoD phosphorylation, although the functional significance of this phosphorylation has yet to be clearly established (Skapek et al., 1995). A basic but controversial question has been whether MyoD in myoblasts is regulated at the level of DNA binding to its target gene promoters. Some reports employing chromatin

immunoprecipitation (chIP) assays have shown that MyoD does indeed occupy the Myogenin promoter in myoblasts, but may be kept inactive by its association with repressive chromatin modifying enzymes such as histone deacetylases (HDACs) or histone methyltransferases (HMTs) (Mal and Harter, 2003; Mal et al., 2001; Mal, 2006). Additional work has built on this model by suggesting that upon cell cycle exit, the hypophosphorylated form of the tumor suppressor protein Rb may titrate HDAC1 away from MyoD to help alleviate repression (Puri et al., 2001). It has not been demonstrated, however, that inhibition of HDACs or HMTs results in significant derepression of myogenic targets in myoblasts, implying that additional regulatory mechanisms are at play (Mal, 2006). Other studies failed even to detect appreciable MyoD recruitment to target promoters under proliferative conditions (Caretti et al., 2004; Serra et al., 2007)

One early finding that supported an alternative model of restricted MyoD DNA binding in myoblasts was the identification of Id proteins. Ids, expressed in growth conditions and downregulated upon differentiation, are helix-loop-helix factors which lack a basic domain and can sequester MyoD and E-proteins into inactive heterodimers (Benezra et al., 1990; Jen et al., 1992). More recent studies have pointed to a role for chromatin structure in controlling MyoD's access to DNA. Multiple reports have demonstrated that myogenic promoters undergo chromatin changes at the level of both histone acetylation and nucleosome remodeling over the course of gene activation. Enzymes involved in both processes, such as p300 and PCAF for acetylation, and the SWI/SNF factors BRG1 and BRM for remodeling, are required for transcriptional induction (de la Serna et al., 2001; Puri et al., 1997). Importantly, Tapscott and colleagues showed that hyperacetylation of histone H4 and recruitment of BRG1 to

myogenic loci appeared to precede the detectable binding of MyoD (by chIP) at these promoters (de la Serna et al., 2005). The implication was that MyoD could only access DNA after such chromatin alterations had already occurred. Additional studies helped formulate an interesting model consistent with the "feed-forward" paradigm in myogenesis, in which MyoD itself helps bring about the chromatin transition required for its own (stable) recruitment. Specifically, it was proposed that MyoD gains access to the Myogenin promoter in two stages, first weakly through association with DNA-bound protein intermediates, and then later directly via E-box DNA occupancy (Berkes et al., 2004). MyoD/E47 was found to physically interact with the homeodomain proteins Pbx1/Meis, constitutively bound factors just upstream of the Mef3 element (Figure 1.4). This interaction requires the same domains within MyoD that are essential for its ability to activate genes within silent chromatin in fibroblasts, suggesting that Pbx1/Meis could serve as a "molecular beacon" for initial MyoD recruitment. Under this model, declining Id protein levels would lead to increased MyoD/E47 association with Pbx1/Meis. Since past work had demonstrated that MyoD physically interacts with acetyltransferases and BRG1 (Puri et al., 1997; Sartorelli et al., 1997; Simone et al., 2004), recruitment of these enzymes would likely accompany association of MyoD and ultimately result in alterations necessary for its stable binding to the E-box (detectable by chIP).

Chromatin structure likely also plays an important role in determining why MyoD activates subsets of target genes in different temporal windows following the initiation of myogenesis. A plausible model posits that target promoters induced at later times may require more extensive chromatin alterations prior to activation, and/or the activities of additional MyoD cofactors. The work of Sartorelli and coworkers, for example, has

demonstrated that in myoblasts, the Polycomb methyltransferase Ezh2 deposits the repressive histone H3-K27-methyl mark specifically on the promoters of late-phase genes, such as myosin heavy chain (MHC) and muscle creatine kinase (MCK) (Caretti et al., 2004). Activation of these late-phase genes presumably requires the activity of a demethylase enzyme to remove this modification and facilitate gene induction. In other work, Tapscott and colleagues have shown that MyoD recruitment to target gene promoters generally tracks with the timing of gene expression, supporting the notion that MyoD DNA binding is rate-limiting (Bergstrom et al., 2002). These studies have suggested that induction of late-phase genes may require the concerted actions of Mef2D and the mitogen-activated protein kinase (MAPK) p38, a molecule that promotes myogenesis through multiple mechanisms, including chromatin remodeling (Penn et al., 2004). Given that Mef2D expression and p38 activity increase over the course of differentiation, these results again point to a model in which MyoD works in tandem with downstream partners to orchestrate a cascade of myogenic gene transcription.

Signaling inputs to the myogenic program

A wide range of signaling cascades has been implicated in the control of embryonic and post-natal myogenesis. Signaling downstream of the MAPK p38 is one of the best characterized. p38 is essential for myogenesis in the embryo and for effective satellite cell function in the adult (de Angelis et al., 2005; Jones et al., 2005). This kinase can be thought of as a master potentiator of myogenesis that positively influences the transcriptional apparatus on multiple, complementary levels. While phospho-p38 (the activated form) has been known for some time to increase over the course of myogenesis,

the mechanism by which this MAPK becomes activated was only recently explored. Work by Krauss and colleagues revealed that the myogenic transmembrane protein CDO, a member of the immunoglobulin superfamily, plays a critical role in p38 activation via the scaffold proteins JLP and Bnip-2, and the Abl tyrosine kinase (Bae et al., 2009; Kang et al., 2008). Initial studies had demonstrated that CDO promotes myogenesis in vitro by enhancing MRF:E protein heterodimer formation via the hyperphosphorylation of E proteins (Cole et al., 2004). Interestingly, CDO was shown to be a target of MyoD, adding yet another layer of positive feedback. Subsequent studies showed that p38 itself was responsible for phosphorylating E47 on serine 140 (Lluis et al., 2005). p38 also directly phosphorylates Mef2A and Mef2C and thereby promotes their transcriptional activity (Wu et al., 2000b). Another important and surprising role for p38 was revealed in its ability to associate with myogenic promoters and help recruit the SWI/SNF chromatin remodeling complex (Simone et al., 2004). Finally, separate work has demonstrated that p38 may also function post-transcriptionally by phosphorylating and inactivating the protein KSRP, which binds to selected myogenic transcripts and directs them to exosome-mediated degradation (Briata et al., 2005).

A second signaling cascade of particular importance in muscle is that initiated by the insulin-like growth factors (IGF1/IGF2). IGFs act through multiple pathways to promote myoblast proliferation, differentiation, and hypertrophy and have been implicated in both muscle development and regeneration (Barton-Davis et al., 1998; Liu et al., 1993; Musaro et al., 2001). The use of pharmacological inhibitors on cultured myoblasts suggested early on that signaling downstream of IGF1 diverges into at least two arms, one of which works through ERK to maintain proliferation, and one of which

acts via PI3K to induce differentiation (Coolican et al., 1997). ERK activity was postulated to be a transient response that declines to allow for cell cycle exit. Signaling downstream of PI3K serves to enhance the transcriptional activities of both MRFs and Mef2 proteins. A recent study has provided evidence that an important target of PI3K, acting via the downstream kinases Akt1 and Akt2, is the coactivator and histone acetyltransferase p300 (Serra et al., 2007). Akt was shown to directly phosphorylate p300, resulting in enhanced physical interaction with MyoD. Past work had demonstrated that p300 facilitates the formation of a trimeric complex with MyoD and the acetyltransferase PCAF, which directly acetylates MyoD to augment its DNA binding (Puri et al., 1997; Sartorelli et al., 1999). PI3K also appears to positively affect Mef2 via phosphorylation, although the actual kinase responsible has not been identified and does not appear to be Akt (Tamir and Bengal, 2000; Xu and Wu, 2000). Interestingly, IGF's hypertrophic effects derive in part from its ability to counteract catabolic pathways via the phosphorylation and inactivation of Foxo transcription factors, known activators of atrophy-promoting E3 ligases (Sandri et al., 2004; Stitt et al., 2004).

While p38 and IGF are both pro-myogenic, they appear to act through independent pathways. Inhibition of PI3K does not affect the normal increase in phospho-p38 that accompanies differentiation, and inhibition of p38 does not influence Akt phosphorylation (Tamir and Bengal, 2000; Wu et al., 2000b). Nevertheless, recent evidence suggests that these signals may ultimately converge or integrate on the chromatin of myogenic target genes. Past work had demonstrated that p38 inhibition blocked chromatin remodeling at these promoters, apparently a consequence of impaired SWI/SNF recruitment (Simone et al., 2004). But interestingly, it has now been shown

that PI3K inhibition also results in compromised chromatin remodeling, despite normal recruitment of SWI/SNF (Serra et al., 2007). This finding suggests that PI3K activity, perhaps due to its requirement for histone acetylation via p300, appears essential for enabling the DNA-associated SWI/SNF enzyme to carry out its biological functions.

Part 2: The Notch signaling pathway

Notch: The core pathway

The Notch signaling cascade has emerged as another critical regulator of in vivo muscle development and regeneration. Notch derives its name from the phenotype of a mutant fly with notched wings, identified in the early 1900s by T. H. Morgan (Mohr, 1919). Only decades later, in 1985, was the Notch locus cloned and characterized in Drosophila by Artavanis-Tsakonas and colleagues and shown to encode a single-pass transmembrane protein (Wharton et al., 1985). Two additional membrane-bound proteins, Delta and Serrate, were cloned shortly thereafter (Fleming et al., 1990; Vassin et al., 1987) and found to physically interact with the Notch protein (Fehon et al., 1990; Rebay et al., 1991). It was quickly appreciated that Notch functions as a cell-surface receptor and mediates cell-cell communication by engaging with its ligands (Delta, Serrate) on neighboring cells to initiate an intracellular signaling cascade.

In mammals, there are four Notch receptors (Notch1-4) and five Notch ligands (Jagged1,2 and Delta-like-1,3,4). Notch exists as a heterodimer on the cell membrane, a result of a cleavage event (S1) mediated by a furin-like protease in the Golgi apparatus (Figure 1.6) (Blaumueller et al., 1997; Logeat et al., 1998). Structurally, the extracellular

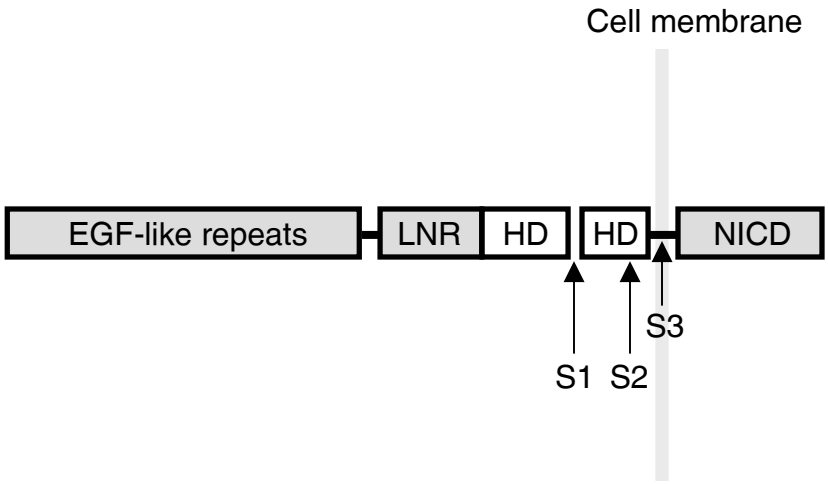

Figure 1.6. Proteolytic cleavages of the Notch receptor. S1 cleavage is mediated by a furin-like protease in the Golgi apparatus to generate the Notch heterodimer. S2 cleavage by ADAM family metalloproteases occurs after engagement of receptor by ligand. S3 cleavage by the γ-secretase complex liberates the Notch intracellular domain (NICD). LNR, Lin12-Notch repeats; HD, heterodimerization domain. Adapted from Gordon et al. (2007).

portion of the receptor is characterized by a series of Epidermal Growth Factor (EGF)-like repeats and three Lin12-Notch repeats (LNR), followed by the heterodimerization (HD) domain. The Notch intracellular domain (NICD) contains two protein-protein interaction motifs (RAM" and ankyrin repeats), a nuclear localization signal, a transcriptional activation domain (in Notch1,2), and PEST motif.

One of the early challenges in the Notch field was to define the mechanism by which ligand-receptor engagement resulted in signal activation within the receptor-expressing cell. It soon became apparent that proteolysis of the receptor itself was an essential aspect of signaling, resulting in the liberation of NICD and its subsequent translocation to the nucleus (Figures 1.6 & 1.7). Early studies that addressed how such proteolysis occurred were facilitated by the use of a truncated form of Notch lacking the extracellular domain, NotchΔE, which for reasons unknown at the time exhibited signaling activity in the absence of ligand stimulation. By transfecting cells with a doubly tagged NotchΔE construct, in which an HA epitope was fused to the N-terminus, and a Myc epitope was fused at the C-terminus, Kopan and colleagues demonstrated that HA reactivity was detectable on the cell surface, while Myc reactivity was localized to the nucleus (Kopan et al., 1996). Struhl and coworkers obtained evidence of NICD liberation and translocation via a different approach, showing that a Notch-Gal4-VP16 fusion construct elicited transcriptional activation only if Gal4-VP16 was placed in the intracellular domain of the receptor (Struhl and Adachi, 1998). Subsequent work used protein microsequencing on receptor fragments to ascertain that a cleavage event occurred at Valine 1744, which lies within the transmembrane domain. Strikingly, mutation of this specific residue of the truncated receptor compromised signaling activity

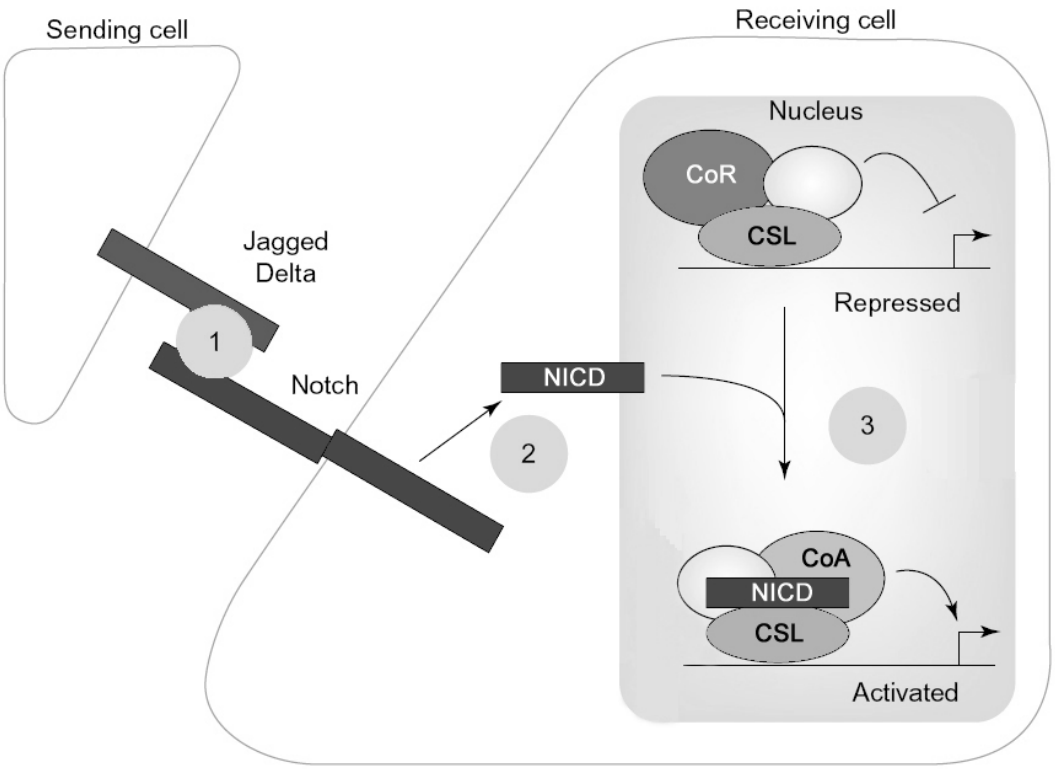

Figure 1.7. The Notch signaling pathway. Engagement of receptor by ligand (1) results in two proteolytic cleavages that liberate the Notch intracellular domain (NICD) and allow for its nuclear translocation (2). In the off state, Notch target genes are bound by the transcription factor CSL, complexed with corepressors. Upon association of NICD with CSL, corepressors are replaced by coactivators, and gene activation occurs (3). Adapted from Kadesch (2004).

as evaluated by a Notch-responsive reporter construct (Schroeter et al., 1998). More significantly, knock-in mice carrying this single mutation in the endogenous Notch1 locus exhibited comparable phenotypes as Notch1 knockout animals (Huppert et al., 2000). While this result initially suggested that cleavage at Valine 1744 was essential for NICD generation, later work revealed that S3 proteolysis can also occur at Leucines 1745-1746 and Serine 1747 (Tagami et al., 2008). The NICD molecules generated from these alternative cleavages, however, are far less stable than NICD-Valine 1744 and apparently cannot compensate for its absence in vivo. Genetic data from both Drosophila and mouse identified presinilin, a component of the γ-secretase complex, as the protease required for S3 proteolysis (De Strooper et al., 1999; Struhl and Greenwald, 1999).

While these studies explained the mechanics of NICD processing, it remained unclear why a truncated Notch receptor allowed for constitutive NICD generation, while the full-length receptor required ligand engagement for activation. Kopan and Israel provided an explanation by discovering an additional cleavage event, S2, that occurs within the extracellular juxtamembrane domain of Notch (Figure 1.6) (Brou et al., 2000; Mumm et al., 2000). Normally inhibited by the presence of the extracellular domain, S2 cleavage only proceeds after a presumed conformational change following ligand-receptor engagement (Gordon et al., 2007). Separate work has established that a critical event in Notch activation is the endocytosis of the Notch ligand (Delta) and trans-endocytosis of the receptor extracellular domain into the signal-sending cell (Itoh et al., 2003; Koo et al., 2005; Parks et al., 2000). It has been speculated that the mechanical force generated by these endocytotic movements may in fact drive the conformational transition that allows for S2 cleavage. Mediated by ADAM-family metalloproteases

(Brou et al., 2000), this processing step precedes and is required for the presinilin-mediated cleavage (S3). Truncation of the receptor alleviates auto-inhibitory control over S2 cleavage, allowing for constitutive production of NICD. Interestingly, treatment of Notch-expressing cultured cells with the calcium chelator EDTA also results in unregulated S2 cleavage and NICD processing. It appears that stability of the Notch heterodimer on the membrane depends on calcium, and its depletion results in dissociation of the extracellular portion of the receptor (Rand et al., 2000).

Genetic studies from fly combined with biochemical assays in cultured cells helped define the molecular events that occur downstream of Notch proteolysis (Figure 1.7) (Fortini and Artavanis-Tsakonas, 1994; Jarriault et al., 1995). Once translocated to the nucleus, NICD associates with the transcription factor CSL (CBF1, Suppressor of Hairless, Lag-1), which typically is constitutively bound to the promoters of Notch-responsive genes. In the absence of ligand stimulation, CSL associates with corepressors. In mammals, these include N-CoR, SMRT, SHARP, and CtIP/CtBP (Kao et al., 1998; Oswald et al., 2002; Oswald et al., 2005), while in flies, the adaptor protein Hairless allows for indirect association with Groucho and CtBP (Barolo et al., 2002). It is thought that CSL-dependent recruitment of these corepressors may play a role in silencing the expression of Notch target genes in the absence of signaling activity. Binding of NICD results in the displacement of these corepressors and the recruitment of coactivators such as Mastermind-like (MAML) and p300 (Oswald et al., 2001; Wu et al., 2000a). Crystal structures of the CSL-NICD-MAML ternary complex revealed that association of the NICD RAM domain with CSL may result in a conformational change important for corepressor displacement, while binding of the NICD Ankyrin repeats to the CSL C-

terminus creates a novel interface for MAML recruitment (Nam et al., 2006; Wilson and Kovall, 2006). Interestingly, recent work from Drosophila has added a new twist to the conventional view of Notch activation. Studies by Bray and colleagues in cultured insect cell lines have demonstrated that at least at a subset of Notch-responsive promoters, CSL DNA binding increases significantly but transiently following EDTA-mediated signal initiation (Krejci and Bray, 2007). While not yet confirmed in vertebrate or ligand-based systems, these results suggest the possibility that NICD may play a role in facilitating CSL recruitment to DNA at specific target promoters.

While much attention has focused on the mechanisms of Notch activation, an equally important question relates to how activity is terminated to ensure proper temporal control of signaling. The work of Jones and coworkers has contributed important insights into this problem by proposing a model in which activation and termination are intimately linked through regulation of NICD protein stability. An intriguing initial observation was that in cells transfected with NICD, CSL, and MAML, less NICD protein was detectable as compared to that seen in cells transfected with NICD and CSL alone (Fryer et al., 2002). Mutation of the NICD PEST domain, a motif known to regulate protein stability, eliminated this effect, as did truncation of the MAML C-terminus. The implication was that MAML, a factor primarily thought of as a transcriptional coactivator, might also contribute to NICD turnover. Indeed, further studies revealed that MAML appears to recruit a specific kinase (CDK8) that phosphorylates the NICD PEST domain and marks it for subsequent ubiquitin-mediated proteasomal degradation (Fryer et al., 2004). These findings suggested a general mechanism also observed in yeast whereby inducible activators such as NICD may

participate in mediating their own destruction to finely limit the temporal widow of their transcriptional activity.

In vivo functions of Notch

Since the cloning of the Notch receptor almost twenty-five years ago, Notch signaling has been shown to play vital roles in the control of cell fate determination in animals ranging from worms to mammals (Artavanis-Tsakonas et al., 1999). One of the first described and most iconic roles for Notch was in mediating a process called "lateral inhibition" in Drosophila (Cabrera, 1990; Chitnis, 1995; Heitzler and Simpson, 1991). In the developing central nervous system of the fly, an initially equivalent group of cells known as a proneural cluster ultimately gives rise to a single neuroblast surrounded by cells of the epidermal lineage. Loss of function mutations in a group of "neurogenic genes", which includes Notch, disrupt this specification process and result in a phenotype of neural hypertrophy. Cells normally fated to the epidermal lineage instead convert to neuroblasts. The implication was that Notch signaling normally inhibits the adoption of the neural fate in the cluster of cells surrounding the single neurally-fated cell. A model of "lateral inhibition" was proposed, in which the single cell destined to become a neuroblast comes to express high levels of Notch ligands but low levels of receptor, rendering it capable of sending but not receiving a Notch signal. Conversely, its neighboring cells acquire the opposite expression profile and exhibit the ability to receive the signal but not initiate it. These expression disparities arise due to the amplification of very small initial differences in the original proneural cluster, resulting from transcriptional feedback effects of signaling on the expression of the receptor and ligands

themselves. Cells experiencing slightly higher initial signaling activity relative to a neighbor, for example, tend to downregulate expression of Notch ligands and upregulate the receptor, while cells with lower initial activity do the opposite. The net result is the eventual clustering of many signal-receiving cells around a single signal-sending cell.

In mammals, Notch signaling regulates the development of a vast array of cell lineages. Genetic knockouts of core Notch pathway components in the 1990s revealed that Notch plays critical early roles in somitogenesis and vascular remodeling during mouse embryonic development (Conlon et al., 1995; Hrabe de Angelis et al., 1997; Xue et al., 1999). Given that animals homozygous null for several of the individual Notch ligands or receptors exhibit early embryonic lethality, conditional deletion approaches have been employed to probe tissue-specific functions of the pathway at later developmental stages. In the immune system, such work demonstrated a genetic requirement for Notch signaling in the specification of T lymphocytes and the generation of Th2 helper T cells (Amsen et al., 2007; Radtke et al., 1999). The pathway's effects on lymphocyte development were also revealed by a gain-of function approach in the case of T-cell commitment (Pui et al., 1999), or via the use of a lineage-specific dominant negative MAML transgenic mouse in the case of Th2 differentiation (Fang et al., 2007). In the skin, deletion of Notch1 results in epidermal hyperplasia, impaired differentiation, and the development of basal-cell carcinoma-like tumors, suggesting tumor suppressor functions (Nicolas et al., 2003; Rangarajan et al., 2001). By contrast, in the nervous system, conditional Notch1 ablation results in premature differentiation of neuroepithelial cells followed by their apoptotic elimination (Lutolf et al., 2002). These studies have highlighted the pathway's functional versatility in different tissues. While in some

contexts, Notch primarily acts to inhibit cellular differentiation of one cell type and perhaps allow for progression down an alternative pathway, in others it actively helps specify or promote the differentiation of a particular lineage. A central challenge has been to understand the molecular circuitry downstream of Notch activation that underlies these diverse biological effects.

Transcriptional effectors of Notch: Enhancer of split/Hes genes

Early genetic studies in Drosophila first illustrated the concept that Notch often controls cell fate decisions through the initiation of a transcriptional cascade, in which primary effectors themselves function as transcriptional repressors that target lineage-determination genes. The inhibitory effects of Notch on the neural fate in the fly were shown to result from the transcriptional induction of a series of basic helix-loop-helix repressor proteins of the Enhancer of Split (E(spl)) locus (Bailey and Posakony, 1995; Jennings et al., 1994; Lecourtois and Schweisguth, 1995). These factors are present in the ectodermal cells surrounding neuroblasts. E(spl) expression is eliminated by loss-of-function mutations in either Notch or Suppressor of Hairless (CSL), while it is expanded under conditions of ectopic Notch activation. The promoters of E(spl) genes contain CSL binding sites which are required for induction (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). Importantly, loss-of-function mutations in genes of the E(spl) locus recapitulate the Notch neural hypertrophy phenotype, with the severity of the hypertrophy dependent upon how many of the genes are mutated (Jennings et al., 1994). Together, these results provided strong evidence that Notch signals through inhibitory E(spl) proteins to restrict neural fate determination.

Further studies suggested that the E(spl) factors and a closely related bHLH protein, hairy, primarily function by repressing the expression of achaete (ac), a proneural bHLH transcription factor. Hairy and E(spl) proteins associate with the Groucho corepressor via a C-terminal WRPW motif and are thought to repress ac transcription via direct DNA binding to an E-box within its promoter (Ohsako et al., 1994; Paroush et al., 1994; Van Doren et al., 1994). This work was quickly extended to vertebrate systems, where a family of several Hairy and Enhancer of Split (Hes) homologues was identified (Sasai et al., 1992). In mammals, the bHLH repressors Hes1, Hes5, and Hes7 are all direct canonical targets of Notch (Figure 1.8) (Bessho et al., 2001; Jarriault et al., 1995; Nishimura et al., 1998). The role of E(spl) proteins in repressing neurogenesis appears to be conserved across species, as the Hes1 knockout mouse exhibits elevated levels of proneural bHLH proteins such as MASH1 (the ac orthologue) and accelerated neurogenesis before perinatal death (Ishibashi et al., 1995). Conversely, retroviral overexpression of Hes1 blocks neural differentiation in vitro and in vivo (Ishibashi et al., 1994). Hes5 partially compensates for Hes1 function in repressing the neural fate, as the Hes1/Hes5 double knockout neural phenotype was more severe than the Hes1 single knockout. Furthermore, forced activation of Notch blocked the differentiation of single knockout, but not double knockout, neural precursors in culture (Ohtsuka et al., 1999).

Transcriptional effectors of Notch: Hey genes

While Hes proteins appear to account for Notch's primary phenotypic effects in the nervous system, these factors by no means represent the full range of Notch transcriptional output. The Hey/Hesr/HERP/CHF family of bHLH repressors (Hey1,

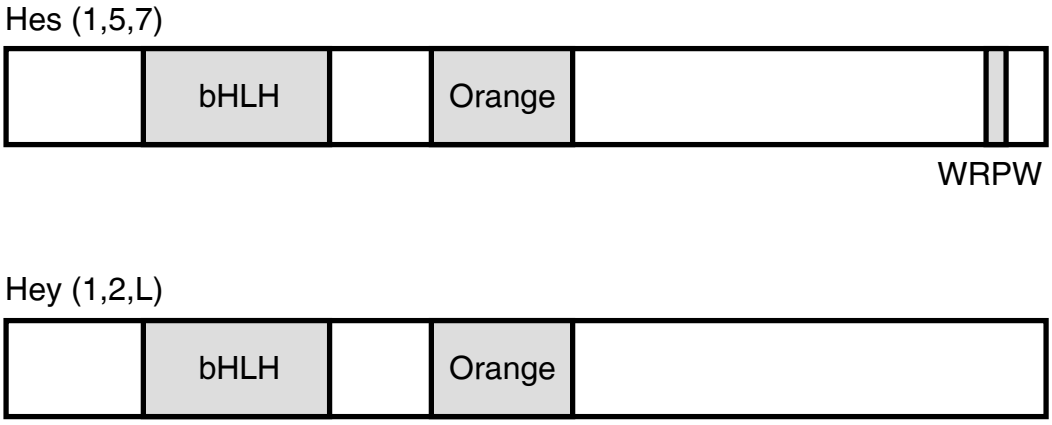

Figure 1.8. Canonical Notch target genes. Hes and Hey transcriptional repressors share a conserved basic helix-loop-helix (bHLH) motif that mediates DNA binding and dimerization, and an Orange domain implicated in protein-protein interactions. Hes proteins contain a C-terminal WRPW tetrapeptide that facilitates recruitment of Groucho/TLE corepressors. Adapted from Iso et al. (2003).

Hey2, HeyL) is another closely related group of transcription factors that are directly activated by Notch in a CSL-dependent fashion (Iso et al., 2002; Iso et al., 2001a; Maier and Gessler, 2000). Hey proteins, like Hes factors, dimerize with one another (and other bHLH proteins) via their HLH motif and bind to E-box or N-box elements within DNA via their basic domain (Figure 1.8) (Fischer et al., 2002; Iso et al., 2001b; Pichon et al., 2004). Hes and Hey proteins also share a conserved Orange domain downstream of the bHLH motif, which is thought to mediate protein interactions and potentially serve as an extended dimerization interface (Taelman et al., 2004). In contrast to Hes family members, Hey factors lack the C-terminal WRPW interaction motif and associate with the corepressors mSin3A, N-CoR, and HDAC1 by way of their basic domain (Iso et al., 2001b).

Gene knockout studies have shown that Hey factors play critical early roles in the developing heart and vasculature and likely represent the prime Notch effectors in these tissues (Fischer et al., 2004; Fischer et al., 2007; Gessler et al., 2002). As with Hes1 and Hes5 in the nervous system, functional redundancy exists among Hey proteins, as single knockout animals are either normal or display less severe phenotypes than double knockouts (Fischer et al., 2004; Fischer et al., 2007). The detailed molecular mechanisms by which Hey proteins function in specific biological contexts are not well defined. While Hey family members are capable of binding to DNA in-vitro, several reports have shown that binding to E-boxes within target promoters either does not occur or is not required for Hey-directed repression (Fischer et al., 2005; Holderfield et al., 2006; Huang et al., 2004; Nakagawa et al., 2000). Instead, Hey proteins often may physically associate with and antagonize the activity of other transcription factors. Hey family members are

capable of binding to GATA4/6 and inhibiting GATA-driven cardiac gene expression, which could partially account for important functions in the cardiovascular system (Fischer et al., 2005). In other contexts, inhibitory physical interactions between Hey proteins and Runx2 may explain Notch-mediated protection against aortic calcification (Garg et al., 2005), while associations between Hey factors and Ptf1-p48 may underlie Notch-directed inhibition of pancreatic exocrine differentiation (Ghosh and Leach, 2006).

Beyond the canonical Hes/Hey target genes, recent studies have revealed that Notch likely induces the expression of a much broader set of genes in various tissues. For example, in helper T-cell differentiation, direct induction of the transcription factor Gata3 downstream of Notch is required for the pathway's ability to promote Th2 specification (Amsen et al., 2007; Fang et al., 2007). In T-cell leukemia and mammary tumorigenesis, by contrast, Notch's oncogenic effects have been linked to the direct induction of c-myc (Klinakis et al., 2006; Weng et al., 2006). And in skin, Notch-mediated upregulation of the cell cycle inhibitor p21 is required for the pathway's growth suppressive effects (Rangarajan et al., 2001). The Notch-E(spl)-ac axis from Drosophila provides an appealingly simple illustration of how Notch influences cell fate, but clearly represents only one of many possible signaling modules employed by the pathway to bring about its diverse biological effects.

Modulation of the Notch signal

A large number of regulatory controls have evolved to restrict and finely tune Notch activity and ensure a spatially and temporally appropriate signaling response. These modulators function at all levels of the pathway, from ligand-receptor engagement,

to receptor processing, to NICD stability and activity (Kadesch, 2004; Kopan and Ilagan, 2009). Two of the earliest identified modulators were the glycosyltransferase enzyme Fringe and the cytoplasmic protein Numb. Work in Drosophila originally demonstrated that Fringe potentiates the response of Notch-expressing cells to the ligand Delta, but inhibits responsiveness to Serrate (Panin et al., 1997). This effect was only observed if Fringe was expressed in the cells expressing the Notch receptor. Later studies showed that Fringe acts in the Golgi as a glycosyltransferase to elongate O-linked fucose residues on the EGF repeats of Notch (Bruckner et al., 2000; Hicks et al., 2000; Moloney et al., 2000). This post-translational modification of the receptor is thought to influence the efficiency with which different ligands can engage receptor and stimulate NICD proteolytic release. Three orthologues of this enzyme exist in mammals (lunatic fringe, radical fringe, and manic fringe), all of which exhibit similar glycosyltransferase activity (Moloney et al., 2000).

Numb was also characterized initially in the fly, where it was shown to be a membrane-associated protein that segregates asymmetrically into one daughter cell of a dividing sensory organ precursor in the peripheral nervous system (Rhyu et al., 1994). $Numb^{+}$ cells were shown to adopt the neural fate, as would be expected of cells which turn off the inhibitory Notch pathway. It was later demonstrated that Numb physically associates with the intracellular domain of membrane-bound Notch and prevents NICD nuclear translocation (Wakamatsu et al., 1999). Ubiquitin-mediated degradation and receptor endocytosis have been proposed as potential mechanisms for Numb-dependent Notch downregulation (Berdnik et al., 2002; McGill and McGlade, 2003). Numb also functions in Drosophila myogenesis (Baylies et al., 1998). In this lineage, Notch first acts

via lateral inhibition in the singling out of a myogenic progenitor cell from a cluster of embryonic mesodermal cells. This progenitor then undergoes asymmetric cell division to generate two daughters, an adult muscle precursor ($Numb^{-}$) and a founder myoblast ($Numb^{+}$). Adult muscle precursors (AMPs) remain undifferentiated and can be thought of as the fly analogue of satellite cells. The asymmetric distribution of Numb in the muscle progenitor daughter cells is consistent with the known inhibitory functions of Notch on myogenic lineage progression. Furthermore, loss of Numb was shown to result in the formation of two AMPs and loss of muscle founders, while forced Numb expression led to the opposite phenotype (Ruiz Gomez and Bate, 1997). In mammals, whether Numb and its homologue Numb-like always function as antagonists of Notch in vivo has been more controversial. Knockout mice have yielded conflicting phenotypes in the developing central nervous system, with reports either of reduced neural differentiation (expected from increased Notch signaling) or premature neural differentiation (expected from decreased Notch activity) (Petersen et al., 2002; Zhong et al., 2000; Zilian et al., 2001).

Part 3: Regulation of skeletal myogenesis by Notch

In vivo roles of Notch in muscle

Recent studies have revealed that the Notch signaling cascade plays critical roles in the regulation of embryonic and post-natal myogenesis. While it has been known for over a decade that forced activation of Notch in cultured myoblasts inhibits their differentiation (Kopan et al., 1994), newer work has demonstrated that this inhibitory

effect in vitro reflects similar functions during development and regeneration in vivo (Conboy et al., 2003; Conboy and Rando, 2002; Schuster-Gossler et al., 2007; Vasyutina et al., 2007). In post-natal myogenesis, Rando and colleagues first made the important observation that muscle injury results in activation of Notch signaling, as evidenced by increased levels of cleaved Notch1, and that this increased signaling may reflect induction of the Notch ligand Delta-like-1 (Dll1) (Conboy and Rando, 2002). Interestingly, experiments in muscle explants further demonstrated that the Notch pathway inhibitor Numb was asymmetrically segregated in dividing intermediate myogenic progenitors. $Numb^{+}$ daughter cells were found to express lineage progression markers, such as Myf-5 and Desmin, but not the earlier pre-myoblast marker Pax3, while $Numb^{-}$ cells exhibited the opposite expression profile. These data suggested that cessation of Notch activity ($Numb^{+}$) correlated with progression down the myogenic pathway, while sustained signaling ($Numb^{-}$) correlated with a maintenance of the undifferentiated state. Additional studies showed that artificial activation of Notch via retrovirally expressed NICD in primary myoblast cultures resulted in enhanced proliferation, while siRNAs targeting the Notch1 receptor led to compromised proliferation. Together, these findings suggested that activation of Notch signaling following muscle injury promoted the expansion of satellite cells or myogenic precursors and prevented differentiation, while termination of Notch activity allowed for subsequent progression down the lineage pathway.

While this work revealed obvious parallels between vertebrate and Drosophila myogenesis with respect to asymmetric Numb segregation, a key limitation was that Rando and coworkers did not address whether Numb plays an actual causal role in

shutting off Notch activity and permitting differentiation in vivo. Since Numb knockout mice die early in gestation (Zhong et al., 2000), obtaining genetic proof of Numb's importance in this process would require the generation of a conditional muscle-specific mutant. While the correlative evidence for Numb is compelling, other mechanisms could also account for the segregation of Notch activity to one daughter cell of a dividing satellite cell or progenitor. Indeed, the work of Rudnicki has revealed a potential role for asymmetric expression of the Notch ligand Dll1 (Kuang et al., 2007). Dll1 was only marginally detectable in the subset of satellite cells that are thought to exhibit true stem cell potential (permanently Myf-5 negative), but was highly expressed in neighboring sister satellite cells that at one point in their history expressed Myf-5. Notch receptors were expressed in both pools of cells, although Notch3 was enriched in the SC stem cell population. These findings suggest that elevated levels of Notch signaling in less committed satellite (stem) cells could result in part from restriction of ligand expression to neighboring sister cells.

Subsequent Notch gain- and loss-of-function studies in vivo built upon these initial observations (Conboy et al., 2003). Inhibition of Notch signaling via a soluble Jagged ligand was shown to impair regeneration following muscle injury, while conversely, enhanced Notch activation via an antibody specific for Notch1, facilitated the repair process. Intriguingly, the compromised regenerative ability of muscle from aged mice was associated with reduced induction of the Notch ligand Dll1, suggesting a potential defect in pathway activation. Consistent with this notion, forced induction of Notch signaling improved the repair response in these animals. Remarkably, subsequent studies in which the circulatory systems of old and young mice were united via parabiosis

suggested that circulating factors in the blood of young animals could help improve the ability of aged muscle to induce Dll1 and ultimately mount a regenerative response (Conboy et al., 2005). This result argued that compromised muscle regeneration in aged animals may primarily reflect an impaired signaling environment, rather than an intrinsic defect of the resident stem cell pool. Indeed, later work expanded on this notion and revealed that aged muscle also exhibits hyperactivation of the Wnt and TGFβ signaling pathways. Elevated Wnt signaling was postulated to account for increased fibrosis in old muscle, as Wnt activation biased myogenic progenitors towards adopting a fibrogenic fate (Brack et al., 2007). Increased levels of TGFβ, by contrast, were proposed to contribute to the defect in satellite cell proliferation, via Smad3-mediated induction of the cell cycle inhibitors p15, p16, p21, and p27 (Carlson et al., 2008). Interestingly, recent work has suggested that Wnt signaling also functions in later phases of a normal regenerative response to promote myogenic determination (Brack et al., 2008). The precise nature of the crosstalk between these multiple signaling pathways in muscle remains to be determined.

In addition to this work on post-natal myogenesis, other studies employed genetic loss-of-function approaches to demonstrate an important role for Notch in embryonic muscle development. Studies in different vertebrate model systems had shown previously that Notch affects the process of somitogenesis, whether by helping to specify somite borders or to impose anterior-posterior polarity (Lewis et al., 2009). It was not clear, however, whether the pathway also functioned downstream in the actual generation and/or maintenance of skeletal muscle. To circumvent the problem of early embryonic lethality caused by a null mutation in the Notch ligand Dll1, Gossler and colleagues

generated a Dll1 null/hypomorph heterozygote, which survived until birth but was found to exhibit striking defects in the skeletal muscle lineage (Schuster-Gossler et al., 2007). The central phenotype observed in this mouse, a dramatic hypotrophy of muscle tissue, appeared to reflect premature, accelerated muscle differentiation, accompanied by loss of the $Pax3^{+}/Pax7^{+}$ myogenic progenitor cell pool. A transient excess of myogenic cells was generated early on in the embryo, but myogenesis terminated too soon to allow for the ultimate generation of normal musculature. The conclusion was that Dll1-Notch signaling is essential for maintaining myogenic progenitors and preventing their precocious differentiation. Using a different approach, conditional CSL knockout mice, Birchmeier and coworkers reported similar findings (Vasyutina et al., 2007). Genetic deletion of CSL in either somitic or migrating myogenic precursors resulted in premature loss of these progenitors due to enhanced early differentiation. An absence of satellite cells as assessed by electron microscopy and Pax7 staining was also observed in these animals, consistent with previous proposals that the $Pax3^{+}/Pax7^{+}$ pool of progenitors represents the cellular origin of this stem cell population.

Mechanisms of Notch-mediated inhibition of myogenesis

While Notch carries out important functions in embryonic and post-natal skeletal myogenesis, the molecular mechanisms by which Notch exerts its effects are not well understood. In the last fifteen years, the question of how Notch represses muscle differentiation has been tackled by a number of different studies but has remained controversial and resistant to solution. In 1994, it was first observed by Weintraub and colleagues that forced expression of NICD repressed myoblast differentiation and

fibroblast conversion in culture (Kopan et al., 1994). This inhibitory effect was also observed upon ligand-mediated activation of the pathway, in which myoblasts were co-cultured with Jagged-expressing cells (Lindsell et al., 1995). The block to differentiation imposed by NICD could be overcome by expression of a MyoD-VP16 fusion protein but not a MyoD~E47 tethered dimer (Kopan et al., 1994). These initial results suggested that NICD might target the transcriptional activity of MyoD without affecting its dimerization or DNA-binding. Such conclusions, however, rested upon the use of an artificial MyoD fusion construct and did not directly assess MyoD occupancy at target gene promoters. Subsequent findings led to an alternative model of Notch action in muscle, whereby Notch would signal through the transcriptional repressor protein Hes1. NICD was shown to directly activate the Hes1 promoter via CSL (Jarriault et al., 1995), and forced expression of Hes1 phenocopied the inhibitory effects of NICD on fibroblast conversion (Sasai et al., 1992). It appeared that Hes1 functioned by disrupting the in vitro DNA binding of MyoD/E47 heterodimers, likely via inactive dimer formation with E47. While initially appealing, this model relied on the use of NICD overexpression and very high levels of transiently transfected Hes1. Indeed, later work by Weinmaster and colleagues demonstrated that Jagged1-mediated Notch signaling in C2C12 myoblasts repressed myogenesis without inducing Hes1 transcription (Shawber et al., 1996). Another study indicated that the Notch ligand Dll1 may in fact induce Hes1, albeit transiently and in an oscillating pattern of expression (Kuroda et al., 1999). Stable myoblast lines expressing Hes1, however, exhibited normal differentiation (Shawber et al., 1996), calling into question the significance of Hes1 induction. In vivo data also revealed that myogenesis was not perturbed in Hes1 knockout animals (Ishibashi et al., 1995).

In parallel with their work on Hes1, Weinmaster and coworkers proposed an alternative theory for Notch-mediated inhibition, arguing for the existence of a CSL-independent pathway. This idea was generated from the observation that mutant forms of NICD that were unable to interact with CSL or activate CSL-dependent gene expression were nevertheless capable of repressing myogenesis in C2C12 cells (Shawber et al., 1996). Furthermore, forced expression of a dominant-negative CSL construct failed to rescue myogenic inhibition imposed by Notch ligands (Nofziger et al., 1999). This proposal was complicated, however, by the fact that residual CSL activity may have persisted in the presence of the dominant negative, and the mutant forms of NICD may still have retained a low-level ability to associate with and activate CSL (Kato et al., 1997). Finally, the in vivo studies described previously revealed very similar phenotypes in the Dll1 null/hypomorph as compared to the conditional CSL knockout, indicating that CSL-dependent signaling accounts for the phenotypic effects of Notch activation, at least in embryonic muscle development (Schuster-Gossler et al., 2007; Vasyutina et al., 2007).

Other reports speculated that Notch may target the Mef2C transcription factor or the p38 signaling pathway, instead of directly antagonizing the muscle regulatory factors. In one study, a physical association was reported between the ankyrin repeats of NICD and Mef2C, which was proposed to account for NICD-mediated inhibition of Mef2C DNA binding and transcriptional activity in reporter assays (Wilson-Rawls et al., 1999). No attempts were made, however, to verify such an association downstream of ligand-mediated signaling. Subsequent work reported that MAML can serve as a coactivator for Mef2C and hypothesized that Notch signaling, via NICD, could titrate MAML away from Mef2C to shut down myogenesis (Shen et al., 2006). While an intriguing idea, it

remains uncertain that MAML is limiting within the nucleus. MAML KO animals exhibited muscle defects and died within ten days of birth, but heterozygotes were phenotypically normal. Further, actual physical interaction between Mef2C and MAML was not demonstrated, and coIPs did not show disruption of NICD-MAML association by Mef2C. Finally, a third study revealed that forced expression of NICD induces expression of MKP-1, a phosphatase that targets p38 for inactivation (Kondoh et al., 2007). While constitutive expression of MKP-1 was indeed shown to block differentiation and reduce phospho-p38, the effects of NICD itself on p-p38 were considerably more modest, and ligand-mediated signaling was not examined.

In summary, while much has been published on Notch and myogenesis, the mechanisms by which this important pathway inhibits myogenic differentiation remain controversial and require further elucidation. To this end, I have sought to define the key transcriptional targets activated by Notch in muscle and explore how these effectors function to inhibit the myogenic transcriptional program. In the first half of my work, I performed a gene expression screen to identify genes upregulated by ligand-mediated Notch activity in C2C12 myoblasts and carried out gain- and loss-of-function experiments to ascertain which of these genes are sufficient and/or necessary for myogenic repression. My results demonstrated that Notch induces the expression of over 80 transcripts after six hours of stimulation. At least two of these genes, the canonical effector Hey1 and the novel responsive gene MyoR, were capable of recapitulating the block to myogenic differentiation when constitutively expressed. siRNA knockdown of these factors alone or in combination, however, did not alleviate repression by Notch, suggesting the existence of multiple, potentially redundant pathways. In the second half

of my studies, I focused my attention on the Notch effector Hey1 and employed a variety of biochemical and functional assays to help uncover the mechanism by which this protein interferes with myogenic transcription. My results indicated that Hey1 does not target the intrinsic transcriptional activity of the skeletal muscle master regulator MyoD, but rather associates with the promoter regions of two critical myogenic targets, Myogenin and Mef2C, to silence their expression.

Chapter II. Inhibition of Myogenesis by Notch: Evidence for Multiple Pathways[1]

ABSTRACT

Notch signaling is critical for skeletal muscle development and regeneration, permitting the expansion of progenitor cells by preventing premature differentiation. I have interrogated the pathways through which ligand-mediated signaling inhibits myogenesis by identifying Notch-responsive genes and assessing their impact on differentiation in vitro. Notch activation led to the robust induction of the transcriptional repressors Hey1 and HeyL in myoblasts, but only constitutive expression of Hey1 blocked myogenesis. siRNA-mediated knockdown of Hey1 had no effect on Notch's ability to inhibit differentiation, suggesting the existence of additional, possibly redundant pathways. I identified 82 genes whose expression was activated when C2C12 myoblasts were cultured in the presence of the Notch ligand Dll4. One of these, MyoR, is a novel Notch-responsive gene, whose protein product is known to repress myogenesis in vitro. siRNA-mediated knockdown of MyoR alone, or in combination with Hey1, was also ineffective at rescuing differentiation in the presence of Dll4. My data support a model in which Notch signaling inhibits myogenesis through multiple pathways, two of which are defined by the Notch-responsive genes Hey1 and MyoR.

[1] Portions of this chapter are reprinted with permission from Buas, M.F., Kabak, S., and Kadesch, T., J Cell Physiol *218*, 84-93 (2009).

RESULTS

To study the mechanisms by which Notch signaling inhibits myogenic differentiation, I employed the myoblast cell line C2C12. This cell line was derived almost three decades ago by serial passaging of myoblasts isolated from an adult mouse hind limb muscle 70 hours following crush injury (Blau et al., 1985; Yaffe and Saxel, 1977). C2C12 cells have long been used as an in vitro model system to investigate the molecular regulation of skeletal muscle differentiation. These cells proliferate as myoblasts in high serum, differentiate and fuse into multinucleated myotubes in low serum, and maintain a population of undifferentiated "reserve cells", which have been likened to quiescent satellite cells (Yoshida et al., 1998).

Ligand-induced Notch signaling blocks an early step in myogenesis

To obtain physiological levels of Notch signaling, I exposed cells to Notch ligands. Specifically, I grew cells in the presence of Fc-fusion proteins, containing either the extracellular domain of Delta-like4 (Fc-Dll4) or Jagged1 (Fc-Jag1), adhered to the surface of tissue culture dishes with an anti-Fc antibody (Varnum-Finney et al., 2000). Plating C2C12 myoblasts on Fc-Dll4, but not on a control Fc-fusion protein (Fc linked to a portion of the Trail receptor 4; dubbed "Fc-control" throughout), led to a robust generation of the Notch intracellular domain (NICD) (Figure 2.1A). When transferred from high serum (GM, growth medium) to low serum (DM, differentiation medium), cells grown on Fc-Dll4 were unable to form myotubes (data not shown) or to induce the expression of Myogenin, an early marker of myogenesis (Figure 2.1A). Similar results

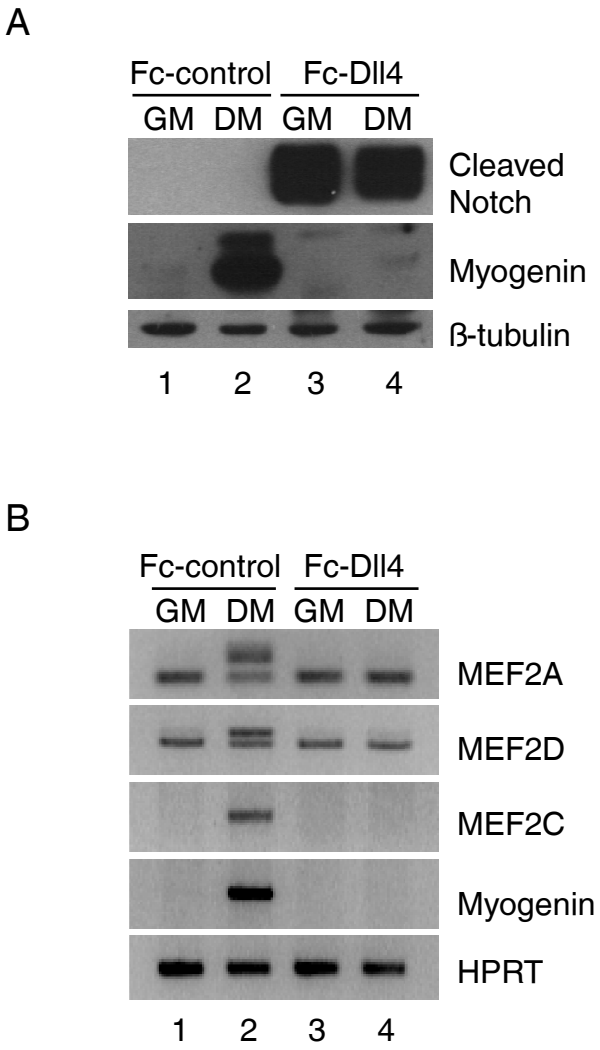

Figure 2.1. Ligand-induced Notch signaling blocks myogenesis. 6-well plates were coated with 4.5 ml of ligand-containing supernatant per well. C2C12 cells were grown on Fc-Dll4-coated or Fc-control-coated plates and switched from growth medium (GM) to differentiation medium (DM) as indicated. Cells were analyzed after 24 hours for (A) cleaved Notch1 and Myogenin proteins (Western immunoblot) and (B) MEF2A, MEF2D, MEF2C and Myogenin RNAs (RT-PCR). The upper bands of the MEF2A and MEF2D RNA doublets are the differentiation-induced splice variants. ß-tubulin protein and HPRT were used as loading controls. Figure 2.1A was contributed by Dr. Shara Kabak.

were obtained using Fc-Jag1 as a Notch ligand. C2C12 cells whose differentiation is blocked by Notch signaling remain myoblasts since they retain the ability to form myotubes when transferred to normal culture dishes (data not shown).

Notch signaling also blocked the induction of RNAs encoding Mef2C and the splicing isoforms of Mef2A and Mef2D normally induced upon muscle differentiation (Figure 2.1B; (Zhu et al., 2005)). Expression of MyoD RNA was reduced in cells exposed to Notch ligand, but a significant level (30-40% of that observed in cells plated on Fc-control) still remained (Figure 2.2A). Additionally, Myf-5 RNA levels were induced by approximately three-fold in cells grown on Fc-Dll4 (Figure 2.2B). These data confirm the results of others that ligand-mediated Notch signaling blocks myogenesis (Lindsell et al., 1995), but challenge the notion that down-regulation of MyoD expression is the primary mechanism responsible for this inhibition (Kuroda et al., 1999). They also suggest that direct antagonism of MEF2C activity by NICD (Wilson-Rawls et al., 1999) is less likely to be important, as Notch signaling acts prior to the induction of Mef2C RNA. Our results indicate instead that Notch functions by repressing the ability of MyoD to induce Myogenin and Mef2C, two critical early mediators in the myogenic program (Cheng et al., 1993; Wang et al., 2001).

Constitutive expression of Hey1 recapitulates the early block to myogenesis

I next determined if members of the Hey or Hes family of transcriptional repressors might mediate the effects of Notch in C2C12 cells. I found that while all three Hey family members (Hey1, Hey2 and HeyL) were induced as a consequence of Notch signaling, the overall level of Hey2 was extremely low (Figure 2.3), consistent with a

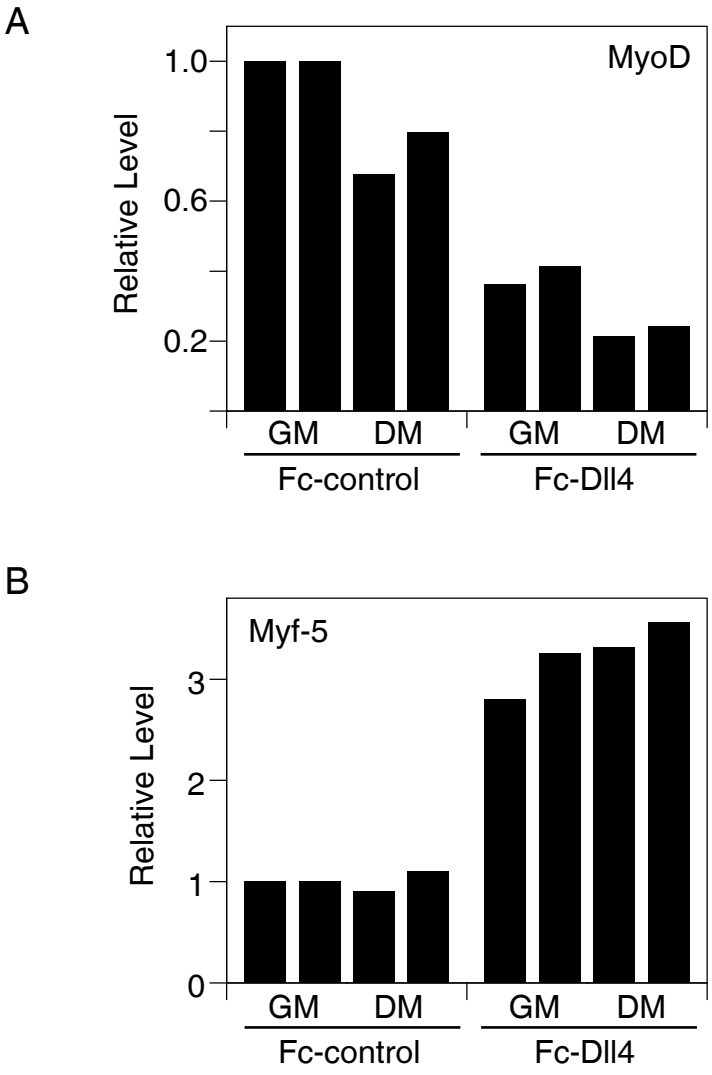

Figure 2.2. Effect of ligand-induced Notch signaling on MyoD and Myf-5 expression. C2C12 cells were grown on Fc-Dll4-coated or Fc-control-coated plates and switched from growth medium (GM) to differentiation medium (DM) as indicated. Cells were analyzed after 24 hours for (A) MyoD and (B) Myf-5 RNAs by quantitative RT-PCR. MyoD and Myf-5 levels are normalized to the Fc-control-GM condition (defined as 1). Data from two replicate samples are plotted.

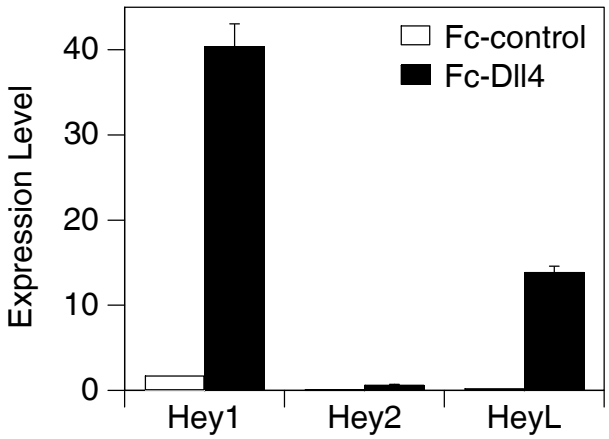

Figure 2.3. Ligand-induced Notch signaling significantly induces Hey1 and HeyL expression. 6-well plates were coated with 3 ml of ligand-containing supernatant per well. C2C12 cells were plated on Fc-Dll4 or Fc-control ligand and propagated for 48 hours in growth medium (GM). Hey1, Hey2 and HeyL RNA levels were determined by quantitative RT-PCR using 18S as a loading control. Expression levels (x) for individual genes were computed from ΔCt values (relative to 18S) according to the formula (x) * $2^{\Delta Ct}$ = (c), where c is an arbitrary constant, and plotted as the average +/- standard deviation of three replicate samples.

previous report (Iso et al., 2001a). Members of the Hes family regulated by Notch in other cell types (Hes1, Hes5, Hes7) were not appreciably induced (Shara Kabak, unpublished observation). To determine if constitutive expression of either Hey1 or HeyL could mimic the effect of Notch on differentiation, I used retroviral vectors to express FLAG-tagged versions of these repressors in C2C12 cells. Cells expressed comparable amounts of Hey1/HeyL RNAs; however, when I assessed differentiation, only Hey1 blocked induction of Myogenin and Mef2C transcripts (Figure 2.4A) and reduced myoblast fusion (data not shown). Western analysis demonstrated that FLAG-Hey1 and FLAG-HeyL proteins were indeed expressed (the indicated bands migrated at the expected mobility of 40-45 kD), albeit at a low level in the case of Hey1 (Figure 2.4B). Induction of both Myogenin and Myosin heavy chain (MHC) proteins occurred normally in the presence of constitutively expressed HeyL, whereas in Hey1-expressing cells, these markers were completely repressed at early time-points and only became detectable by day 4. My results are consistent with a previous report implicating Hey1 as an inhibitor of myogenesis (Sun et al., 2001). While I also observed that a FLAG-tagged version of Hey2 exhibited the ability, like Hey1, to inhibit Myogenin induction (Figure 2.5), I do not consider Hey2 a major player in this system, given that ligand-mediated stimulation induces only a negligible amount of Hey2 transcription. Fc-Dll4 also induced Hey1 in primary human myoblasts, and this correlated with a block to Myogenin induction (Figure 2.6). I conclude that constitutive expression of Hey1 strongly inhibits early inductive events of myogenesis and ultimately delays the course of differentiation.

I next asked if Hey1 induction is necessary for Notch to inhibit myogenesis. I reasoned that if I sufficiently reduced the level of Hey1 expression I might observe

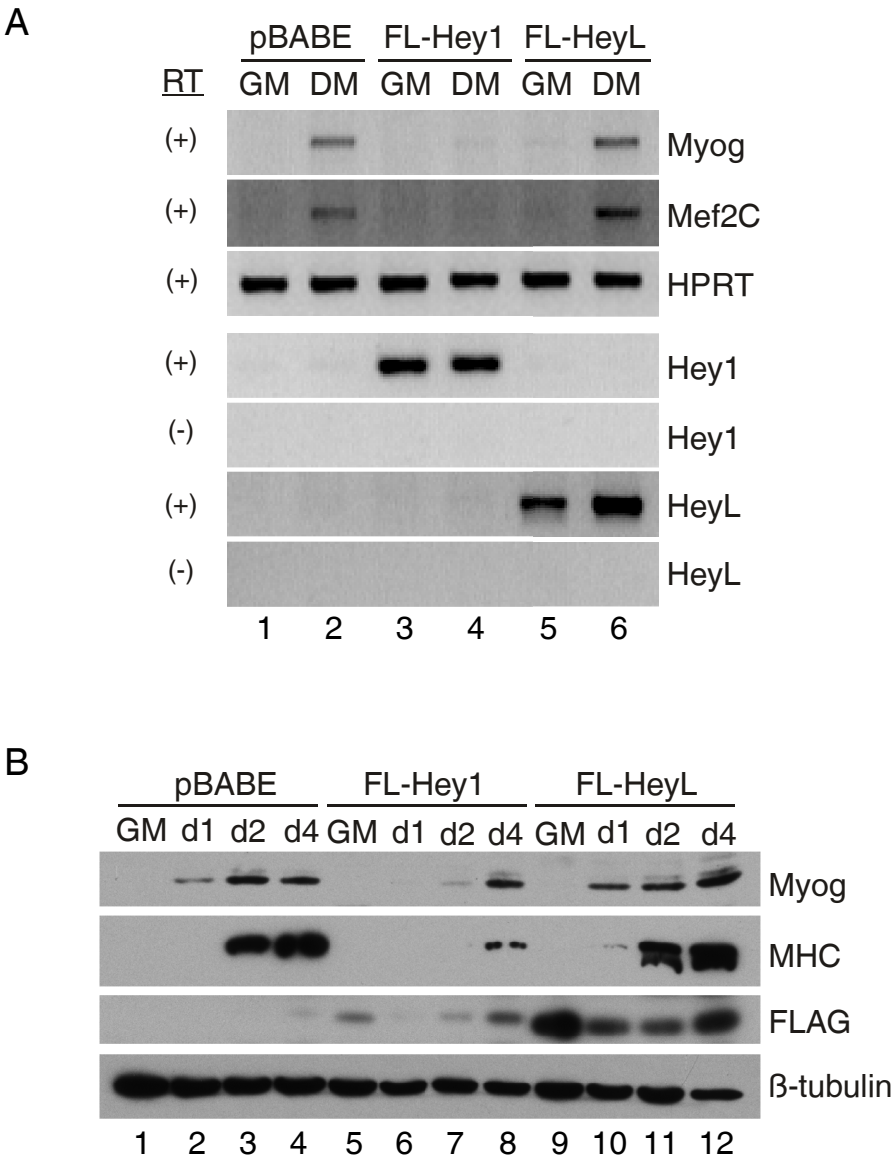

Figure 2.4. Constitutive expression of Hey1, but not HeyL, blocks myogenesis. (A) C2C12 cells were stably transduced with parental retrovirus (pBABE-puro) or retroviruses expressing FLAG-tagged Hey1 or HeyL, propagated in growth medium (GM), shifted to differentiation medium (DM), and analyzed for expression of the indicated cDNAs by RT-PCR, using HPRT as a loading control. RT, reverse transcriptase. (B) Transduced cells were propagated in GM, shifted to DM and analyzed for expression of Myogenin, MHC or FLAG-tagged proteins after 1, 2, or 4 days by Western immunoblotting, using ß-tubulin as a loading control.

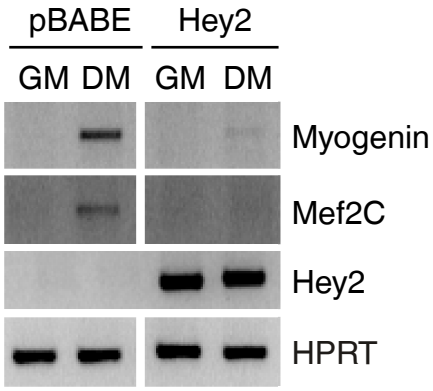

Figure 2.5. Constitutive expression of Hey2 inhibits myogenesis. C2C12 cells were stably transduced with parental retrovirus (pBABE-puro) or a retrovirus expressing FLAG-tagged Hey2. Lines were propagated in growth medium (GM) and then shifted to differentiation medium (DM) for 24 hours and analyzed for expression of Myogenin, Mef2C, and Hey2, by RT-PCR using HPRT as a loading control.

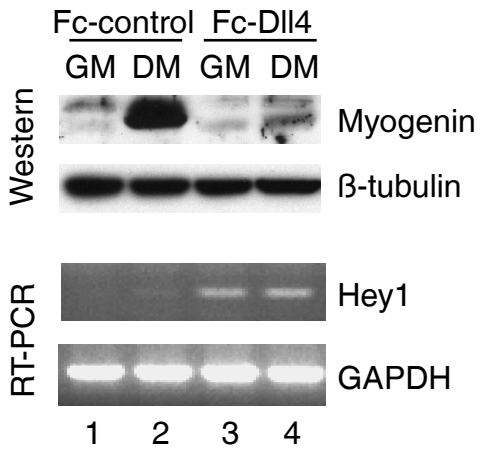

Figure 2.6. Ligand-mediated Notch signaling induces Hey1 and blocks Myogenin induction in primary myoblast cultures. Human myoblasts were grown on Fc-Dll4-coated or Fc-control-coated plates and switched from growth medium (GM) to differentiation medium (DM) for 24 hours as indicated. Myogenin protein was assessed by Western immunoblotting using ß-tubulin as a loading control, and Hey1 RNA was determined by RT-PCR using GAPDH as a loading control. This figure was contributed by Dr. Shara Kabak.

normal Myogenin induction despite ongoing Notch signaling. Transfection of C2C12 cells with siRNAs directed against Hey1, relative to control siRNAs, did not appreciably affect the low level of Hey1 RNA in cells plated on Fc-control (this was somewhat variable across multiple experiments; see Figure 2.19), but led to a significant reduction (~75 percent) in Hey1 expression when cells were plated on Fc-Dll4 (Figure 2.7, top). However, the induction of Myogenin (Figure 2.7, bottom) and two other myogenic markers, Mef2C and Myosin heavy polypeptide 3 (Myh3) (Figure 2.8), was still inhibited. This result argues that the high levels of Hey1 expression induced by Notch are not necessary for the inhibition of myogenesis, and that other, potentially redundant pathways may contribute.

Identification of novel Notch-responsive genes in C2C12 cells

To identify additional effectors downstream of Notch, I performed a microarray-based expression screen using C2C12 cells. Myoblasts were plated on either Fc-Dll4 or Fc-control ligand and maintained in growth medium (GM) for six hours prior to isolation of RNA for expression profiling. The six-hour time-point was chosen to bias the screen towards the detection of direct (early) targets of the pathway. Activation of Notch was verified by Western blot of protein lysates harvested from a parallel set of cultures using an antibody specific for cleaved Notch (data not shown). RNA was submitted to the Penn Microarray Core Facility for processing on Affymetrix MOE430v2.0 gene chips.

With a false discovery rate set to 0% and a fold change cutoff of two, the microarray identified 82 transcripts that were induced and five transcripts that were repressed by Notch ligand stimulation. The top 30 induced genes are shown in Table 1.

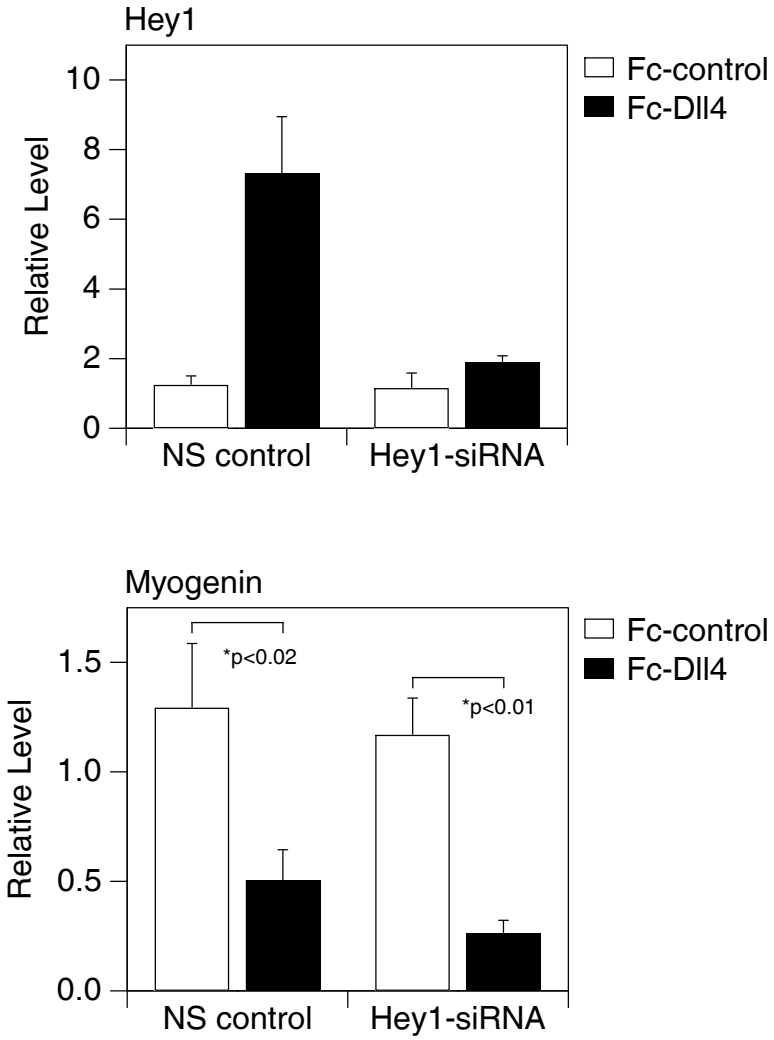

Figure 2.7. Ligand-induced Notch signaling effectively blocks Myogenin induction in cells expressing reduced levels of Hey1. Individual tissue culture wells were treated with Fc-Dll4 or Fc-control as indicated in the Methods. C2C12 cells transfected with either non-silencing (NS) control siRNA oligonucleotides or Hey1-directed siRNAs were propagated on the coated plates and then shifted to differentiation medium (DM) for 24 hours. Expression of Hey1 (upper panel) and Myogenin (lower panel) RNA was assessed by quantitative RT-PCR. Q-PCR values are presented as the average +/- standard deviation of three replicate samples. p values were computed by a standard unpaired t-test.

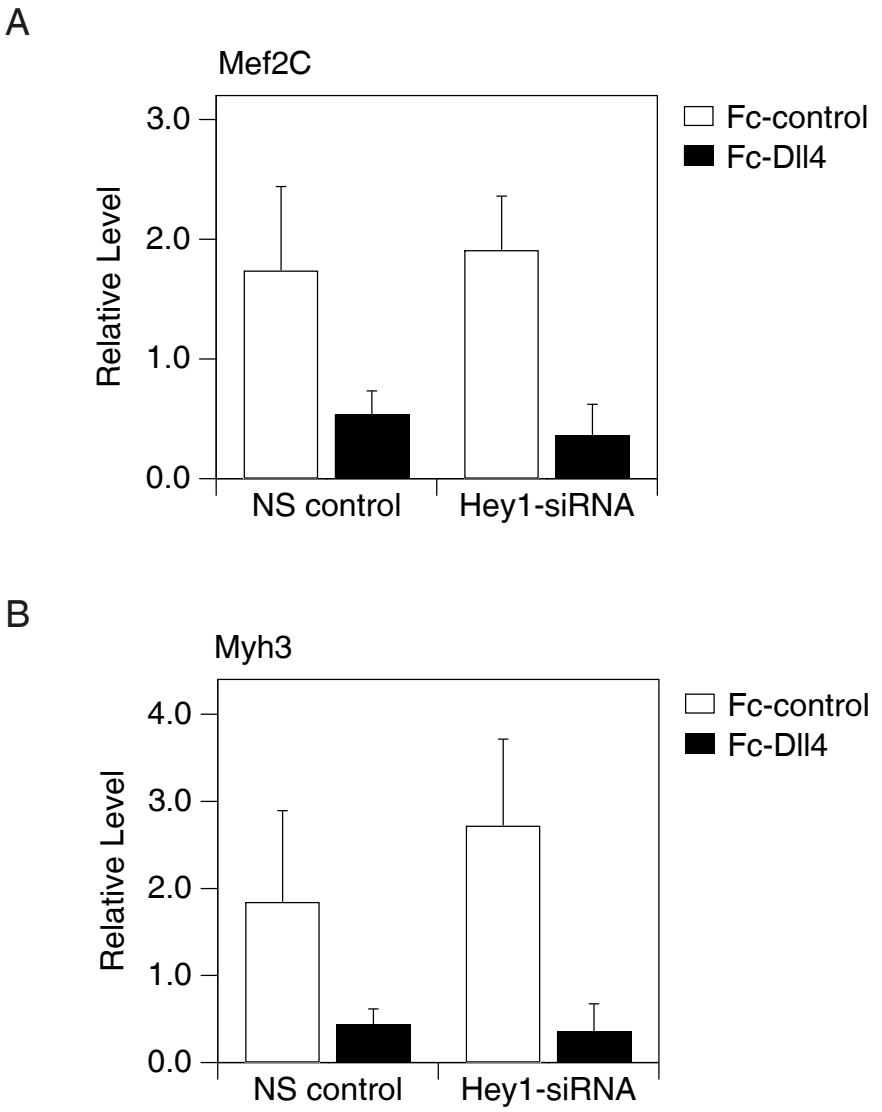

Figure 2.8. Ligand-induced Notch signaling effectively blocks induction of Mef2C and Myh3 in cells expressing reduced levels of Hey1. Individual tissue culture wells were treated with Fc-Dll4 or Fc-control as indicated in the Methods. C2C12 cells transfected with either non-silencing (NS) control siRNA oligonucleotides or Hey1-directed siRNAs were propagated on the coated plates and then shifted to DM for 24 hours. Expression of (A) Mef2C and (B) Myh3 RNA was assessed by quantitative RT-PCR. Q-PCR values are presented as the average +/- standard deviation of three replicate samples. Note: While MHC protein levels were only appreciably induced in control cells by 48 hours in DM, Myh3 RNA was elevated ~300 fold by 24 hours (data not shown).

Table 1. Genes induced by Notch in C2C12 myoblasts.

	Gene Symbol	Fold change[a]		Description
1	**Nrarp**	14.02	[C]	Notch-regulated ankyrin repeat protein
2	**Heyl**	10.86	[C]	hairy/enhancer-of-split related with YRPW motif-like
3	**Hey1**	7.818	[C]	hairy/enhancer-of-split related with YRPW motif 1
4	**Il6**	6.389	[C]	interleukin 6
5	**Msc**	5.204	[C]	musculin [MyoR]
6	**Trib2**	4.982	[C]	expressed sequence AW319517 [Tribbles2]
7	**8430408G22Rik**	4.743	[C]	RIKEN cDNA 8430408G22 [G22]
8	**Ntn4**	4.483	[C]	netrin 4
9	**Dio2**	4.324	[NT]	deiodinase, iodothyronine, type II
10	**Jag1**	4.066	[NT]	jagged 1
11	**BC031353**	3.979	[C]	cDNA sequence BC031353
12	**Myf5**	3.328	[C]	myogenic factor 5
13	**BG143461**	3.242	[NT]	EST mab56d07.x1
14	**Mcf2l**	3.151	[C]	mcf.2 transforming sequence-like
15	**Sec14l2**	3.122	[C]	SEC14-like 2 (S. cerevisiae)
16	**Gata3**	3.104	[C]	GATA binding protein 3
17	**Adora2b**	3.093	[NT]	adenosine A2b receptor
18	**4832420M10**	2.994	[C]	hypothetical protein 4832420M10
19	**Tgfb2**	2.954	[NC]	transforming growth factor, beta 2
20	**Gpr30**	2.927	[C]	G protein-coupled receptor 30
21	**Egfr**	2.923	[C]	epidermal growth factor receptor
22	**4833422C13Rik**	2.85	[NT]	RIKEN cDNA 4833422C13
23	**Vav3**	2.71	[C]	vav 3 oncogene
24	**Cyp1b1**	2.699	[NT]	cytochrome P450, family 1, subfamily b, polypeptide 1
25	**A730054J21Rik**	2.656	[NT]	RIKEN cDNA A730054J21
26	**Kcnf1**	2.618	[NT]	potassium voltage-gated channel, subfamily F, member 1
27	**Epha4**	2.601	[NT]	Eph receptor A4
28	**Calcrl**	2.595	[NT]	calcitonin receptor-like
29	**Id3**	2.579	[C]	inhibitor of DNA binding 3
30	**Ppp1r2**	2.557	[NT]	protein phosphatase 1, regulatory (inhibitor) subunit 2

[a] Targets subsequently confirmed by RT-PCR are marked "C", those not confirmed are marked "NC", and those not tested are marked "NT".

This observed skewing towards gene activation as opposed to repression suggests that our list may indeed contain a large number of direct targets of the pathway, since Notch is primarily thought to function as a transcriptional activator. Three known Notch targets—Hey1, HeyL, and Nrarp—were at the top of the list of induced genes. Among the most highly induced were the cytokine interleukin-6 (IL-6), the transcription factor MyoR, the kinase-like protein Tribbles2 (Trib2), and the RIKEN cDNA 8430408G22 (G22).

I validated a subset of the Notch-responsive genes by RT-PCR. IL-6, MyoR, Trib2, G22, and the known targets Nrarp, Hey1, and HeyL, were all induced to varying extents in C2C12 cells exposed to Fc-Dll4 after six hours (Figure 2.9A). Quantitative RT-PCR provided additional confirmation and revealed fold changes very similar to, or greater than, those reported by the array for Hey1, MyoR, IL-6, and an additional responsive gene, Id3 (Figure 2.9B). In total, 18 of the 30 most highly induced genes were confirmed by RT-PCR, one was not confirmed, and 11 were not tested (Table 1). Further studies demonstrated that the induction of three genes (MyoR, Trib2, and Hey1), was maintained upon serum withdrawal, while Nrarp and IL-6 expression was reduced (Figure 2.10). This result suggests that the expression of certain genes appears to require the combined actions of Notch and undefined factors present in serum.

Expression of a subset of Notch-responsive genes inhibits C2C12 differentiation

I next asked if constitutive expression of any of our newly identified Notch-responsive genes could inhibit myogenesis. I chose to focus initially on a subset of those most highly induced: Nrarp, Trib2, G22, and IL-6. Nrarp, Notch-regulated ankyrin repeat protein, has been shown to function as a feedback inhibitor of the pathway in Xenopus

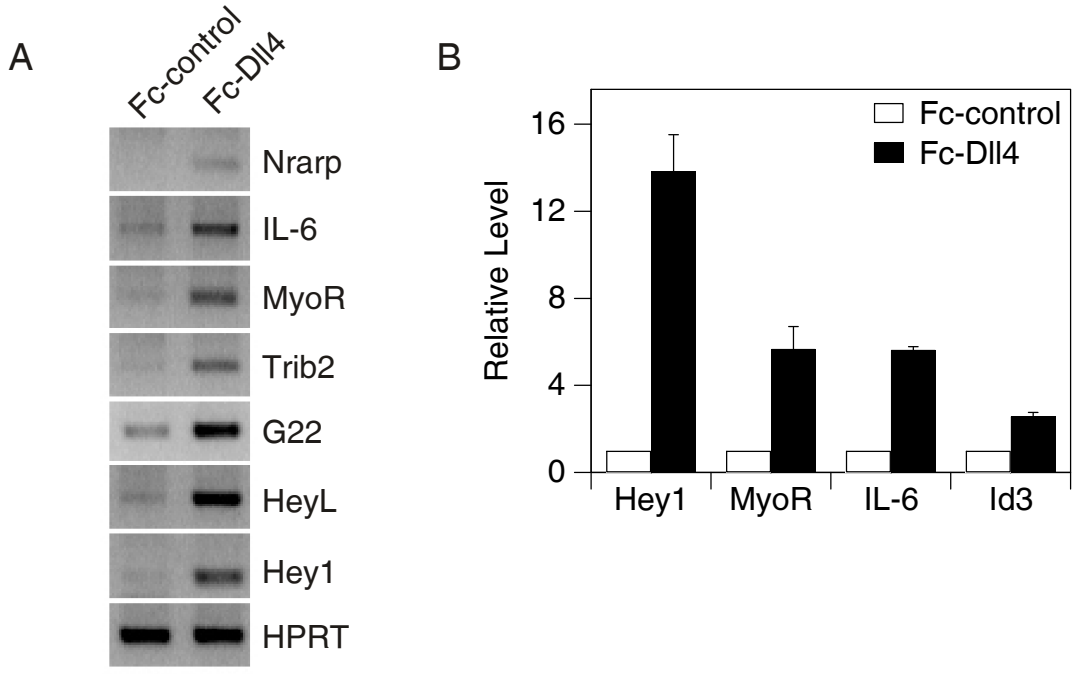

Figure 2.9. Validation of Notch-responsive genes by RT-PCR. 10 cm dishes were coated with 2.5 ml of ligand-containing supernatant. C2C12 myoblasts were plated on either Fc-Dll4 or Fc-control ligand and propagated in growth medium (GM) for six hours. RNA expression of selected genes was determined by (A) RT-PCR or (B) quantitative RT-PCR using HPRT or 18S as a loading control, respectively. Quantitative RT-PCR values for individual genes are normalized to the Fc-control condition (defined as 1) and plotted as the average +/- standard deviation of three replicate samples.

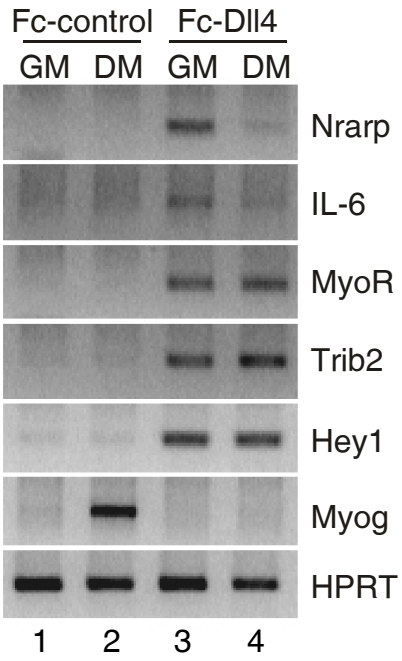

Figure 2.10. Selected Notch-responsive genes are induced by ligand stimulation only in the presence of serum. C2C12 cells were plated on Fc-control or Fc-Dll4 ligand, propagated in growth medium (GM), and switched to differentiation medium (DM) for 24 hours. RNA expression of indicated genes was analyzed by RT-PCR, using HPRT as a loading control.

and zebrafish, but was also reported to augment Notch-mediated transcriptional activation in cultured cells (Ishitani et al., 2005; Lamar et al., 2001). Trib2 is a kinase-like protein implicated in the pathogenesis of acute myelogenous leukemia and also reported to inhibit phosphorylation of Akt, a signaling molecule important in myogenesis (Du et al., 2003; Heron-Milhavet et al., 2007; Keeshan et al., 2006; Naiki et al., 2007; Wilson and Rotwein, 2007). Interleukin-6 is an inflammatory cytokine that is expressed in regenerating muscle and may promote satellite cell proliferation (Cantini et al., 1995; Kami and Senba, 1998).

cDNAs for Nrarp and Trib2 were cloned into the pBABE-puro retroviral expression vector, and individual viruses were used to infect C2C12 myoblasts. Resulting stable cell lines clearly expressed the specified transcripts, but exhibited normal induction of the early differentiation marker Myogenin when deprived of serum for 24 hours (Figure 2.11). Similar results were obtained when cells were infected with a retrovirus expressing the G22 cDNA (data not shown). C2C12 cells were also infected with retroviruses encoding FLAG-tagged Nrarp or Trib2, and protein expression was verified by Western blot (Figure 2.12A). Bands migrating at the appropriate mobility were detected in cells transduced with either of the two retroviruses, while the FLAG-Trib2 signal ran at the same mobility as a non-specific background band (FLAG-Nrarp, ~20 kD (*), FLAG-Trib2, ~45 kD (**)). Induction of Myogenin and MHC proteins occurred normally in FLAG-Nrarp and FLAG-Trib2 cell lines over four days in DM (Figure 2.12B). Fusion of myoblasts into myotubes after three days in differentiation medium was also unimpaired relative to that observed in the pBABE control cultures (Figure 2.12C). Nrarp-transduced cells were also evaluated for their ability to induce Hey1 when

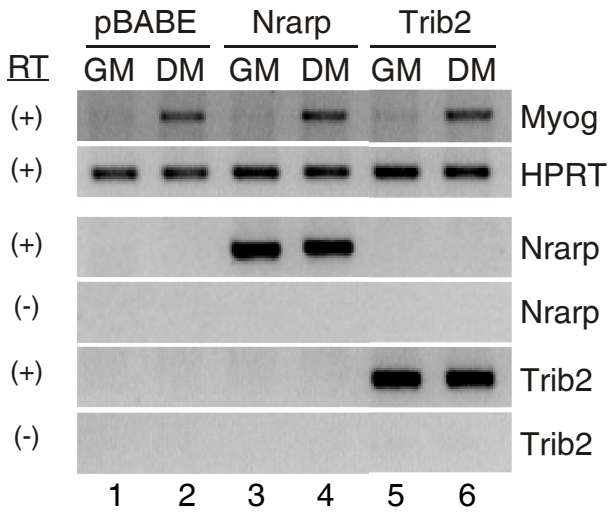

Figure 2.11. Constitutive expression of Nrarp or Trib2 does not inhibit Myogenin induction. C2C12 cells were stably transduced with parental retrovirus (pBABE-puro) or retroviruses expressing Nrarp or Trib2. Lines were propagated in growth medium (GM) and then shifted to differentiation medium (DM) for 24 hours and analyzed for expression of Myogenin or the indicated cDNAs by RT-PCR using HPRT as a loading control. RT, reverse transcriptase.

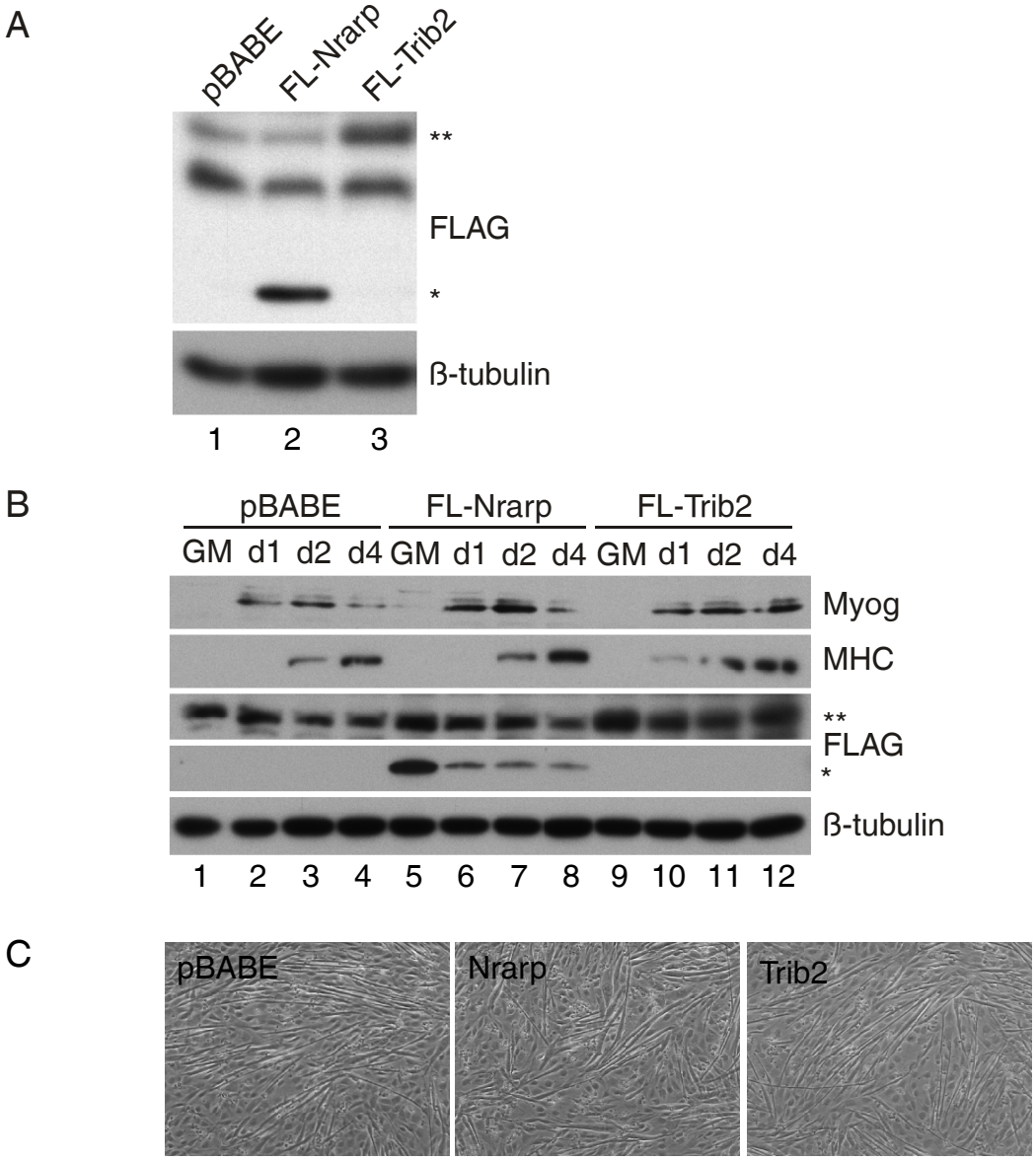

Figure 2.12. Constitutive expression of Nrarp or Trib2 does not impair myogenesis. C2C12 cells were stably transduced with parental retrovirus (pBABE-puro) or retroviruses expressing FLAG-tagged Nrarp or Trib2. (A) Lines were propagated in growth medium (GM) and analyzed for expression of FLAG-tagged proteins by Western immunoblotting. (*) indicates the position of FLAG-Nrarp, and (**) indicates the position of FLAG-Trib2. (B) Transduced cells were propagated in GM, shifted to differentiation medium (DM) and analyzed for expression of Myogenin, MHC or FLAG-tagged proteins after 1, 2, or 4 days by Western immunoblotting using ß-tubulin as a loading control. (C) Fusion of myoblasts into myotubes was examined in the indicated cell lines after three days in DM.

cultured on Fc-Dll4, testing the hypothesis that Nrarp is a feedback inhibitor of Notch signaling. While the induction of Hey1 was moderately reduced, this occurred at a level of Nrarp that exceeded that typically induced by ligand (data not shown). Thus, Nrarp does not appear to exert a significant effect on overall Notch signaling in our system.

I also asked if IL-6 affects myogenesis. I observed a modest dose-dependent inhibition of Myogenin induction after serum withdrawal when cells were bathed in increasing concentrations of IL-6, with maximum inhibition of approximately two-fold occurring at a dose of 100 ng/ml (Figure 2.13). Given that this effect occurred only at high concentrations of the cytokine, which are likely to be supra-physiological, I am hesitant to ascribe a major role to IL-6 in mediating the effects of Notch in muscle, but do not rule out a potential contributory influence.

In contrast to the findings for Nrarp, Trib2, and G22, forced retroviral expression of MyoR resulted in a complete block to C2C12 myogenesis, consistent with a previous report that has implicated this bHLH protein as negative regulator of MyoD and of myogenic conversion of fibroblasts (Lu et al., 1999). MyoR-expressing myoblasts failed to induce Myogenin or Mef2C transcripts at 24 hours after serum withdrawal (Figure 2.14A), showed no induction of Myogenin or MHC proteins over four days in differentiation medium (Figure 2.14B), and exhibited no evidence of fusion after three days in DM (Figure 2.14C). Expression of MyoR in these cells was verified by RT-PCR and Western blot analysis (Figure 2.14A-B). In parallel with these findings, I observed that ligand-mediated Notch signaling also induced expression of ABF-1, the human orthologue of MyoR, in primary human myoblasts (Figure 2.15). I conclude that Notch signaling strongly induces the expression of Nrarp, Trib2, G22, IL-6, and MyoR, but of

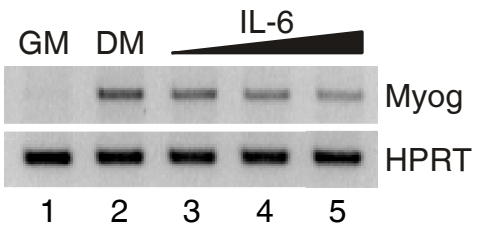

Figure 2.13. IL-6 inhibits myogenesis at high doses. C2C12 cells were maintained in growth medium (GM) and then switched to differentiation medium (DM) for 24 hours in the absence or presence of increasing concentrations of IL-6 (2.8, 10 and 100 ng/ml). Myogenin RNA levels were assessed by RT-PCR using HPRT as a loading control.

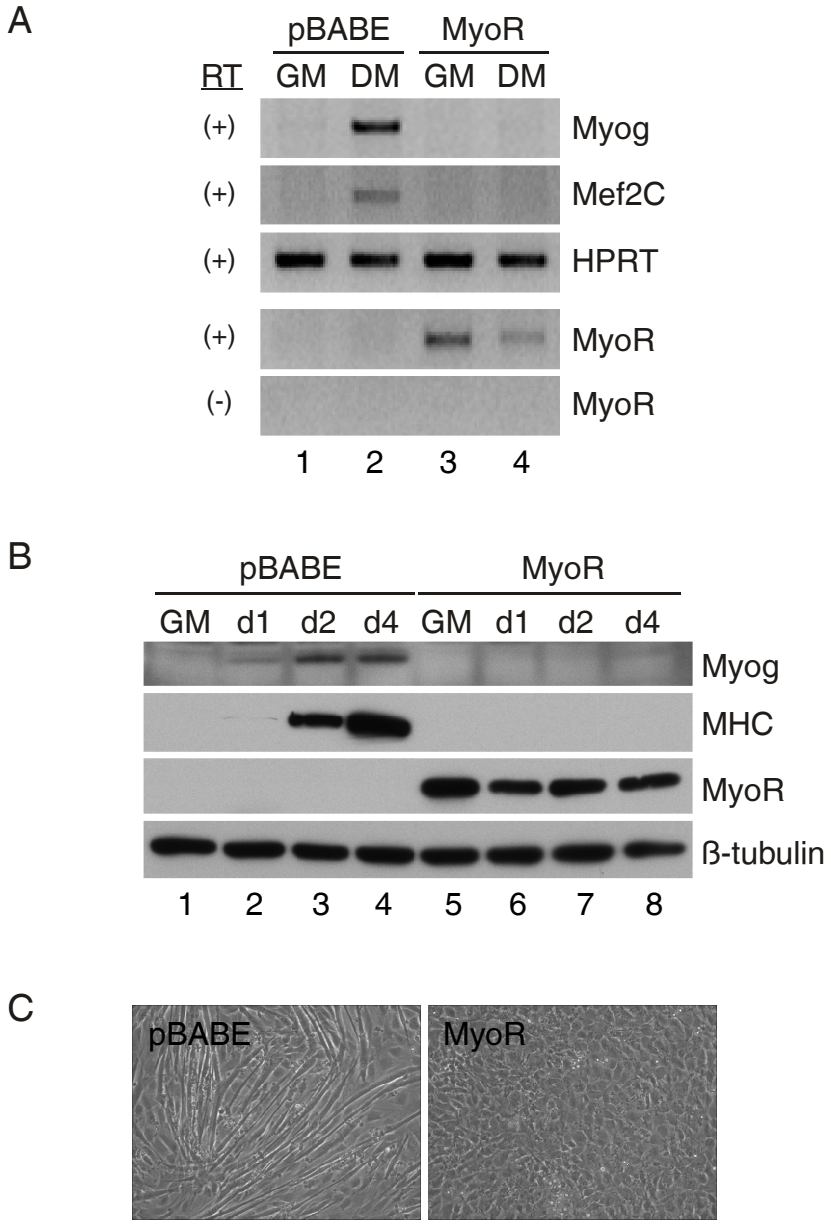

Figure 2.14. Constitutive expression of MyoR inhibits myogenesis. C2C12 cells were stably transduced with parental retrovirus (pBABE-puro) or a MyoR-expressing retrovirus (pBABE-MyoR), propagated in growth medium (GM) and then shifted to differentiation medium (DM). Expression levels of the indicated differentiation markers and MyoR were determined by (A) RT-PCR, after 24 hours in DM or (B) Western immunoblot, after 1, 2, or 4 days in DM. RT, reverse transcriptase. (C) Myoblast fusion was examined in pBABE control cells or MyoR-expressing cells after three days in DM.

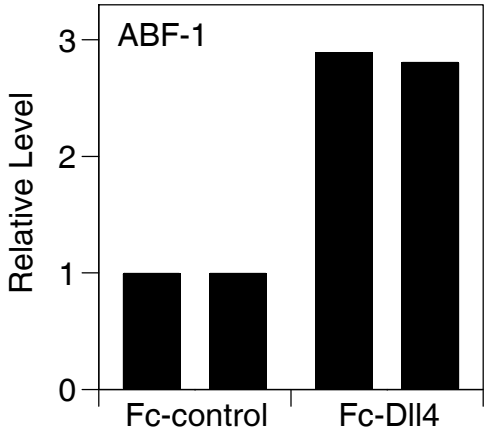

Figure 2.15. Ligand-mediated Notch signaling induces ABF-1 (human MyoR) in primary myoblast cultures. ABF-1 RNA was assessed by quantitative RT-PCR in human myoblasts plated on Fc-Dll4 or Fc-control and propagated in growth medium (GM). Data from two independent myoblast isolations are plotted. Primary cultures were prepared by Dr. Shara Kabak.

these, only constitutively expressed MyoR is sufficient to recapitulate the inhibition imposed by the pathway as a whole.

The precise manner by which MyoR expression is activated by Notch signaling remains an open question. Importantly, I have excluded the possibility that MyoR induction occurs downstream of Hey1, as MyoR RNA levels were essentially unchanged under conditions of Hey1 retroviral expression (data not shown). Bioinformatic analysis failed to reveal any conserved CSL binding sites within the MyoR 2kb proximal promoter, which was found to be unresponsive to NICD in luciferase reporter assays, but a far-upstream potential enhancer region containing three conserved CSL sites was responsive to NICD. However, mutation of these sites did not compromise NICD-responsiveness (Figure 2.16). Accordingly, MyoR could well be an indirect target of Notch, but further studies will be needed to clearly define its mode of regulation.

Knockdown of MyoR alone or in combination with Hey1 does not impair Notch activity

Given that constitutive expression of either Hey1 or MyoR mimicked the inhibitory effects of Notch in C2C12 cells, it appeared that Notch was acting through multiple pathways, and that perhaps no single gene target would be required for Notch to exert repression. To further test this notion, I performed an additional siRNA knockdown experiment to reduce the level of MyoR. Transfection of C2C12 cells with MyoR siRNAs, relative to control siRNAs, led to a significant reduction (>90 percent) in MyoR expression when cells were plated on Fc-Dll4 (Figure 2.17, top). In this experiment, a higher dose of Fc-Dll4 supernatant was employed relative to that depicted in Figure 2.7

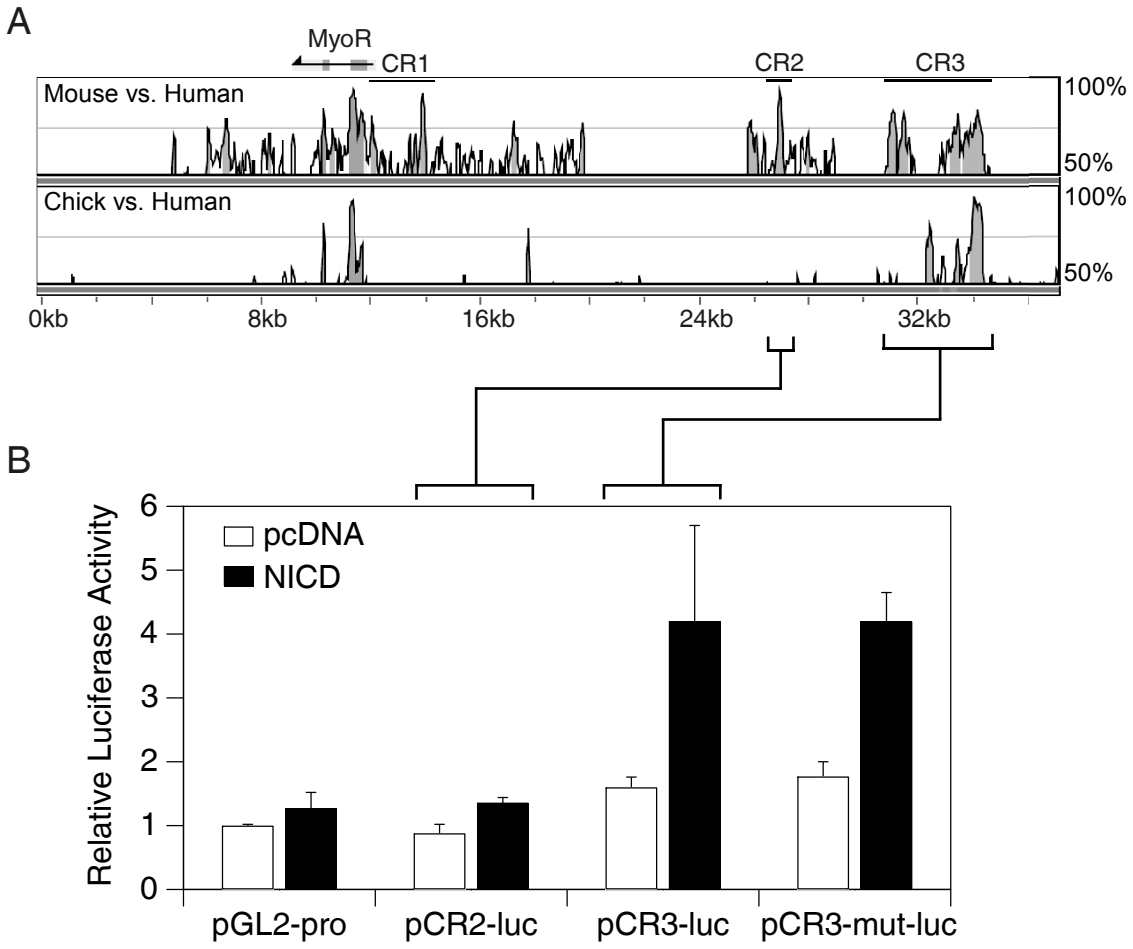

Figure 2.16. A 3.3kb conserved region upstream of the MyoR transcriptional start site shows responsiveness to NICD. (A) VISTA plot depicting conservation between human, mouse, and chicken sequences in a ~36 kilobase region encompassing the MyoR locus. MyoR is a two-exon gene transcribed from right to left. Conserved region 1 (CR1), CR2, and CR3 correspond to elements that were cloned and tested for induction by NICD in reporter assays. Three conserved CSL binding sites identified in CR3 were mutated to generate pCR3-mut-luc. (B) C2C12 cells were transfected with the indicated reporters (2.5 ng) in the absence (pcDNA) or presence (NICD) of an NICD expression vector (25 ng). Values are normalized to the activity of a co-transfected Renilla luciferase vector and presented as averages of three replicate samples +/- standard deviation.

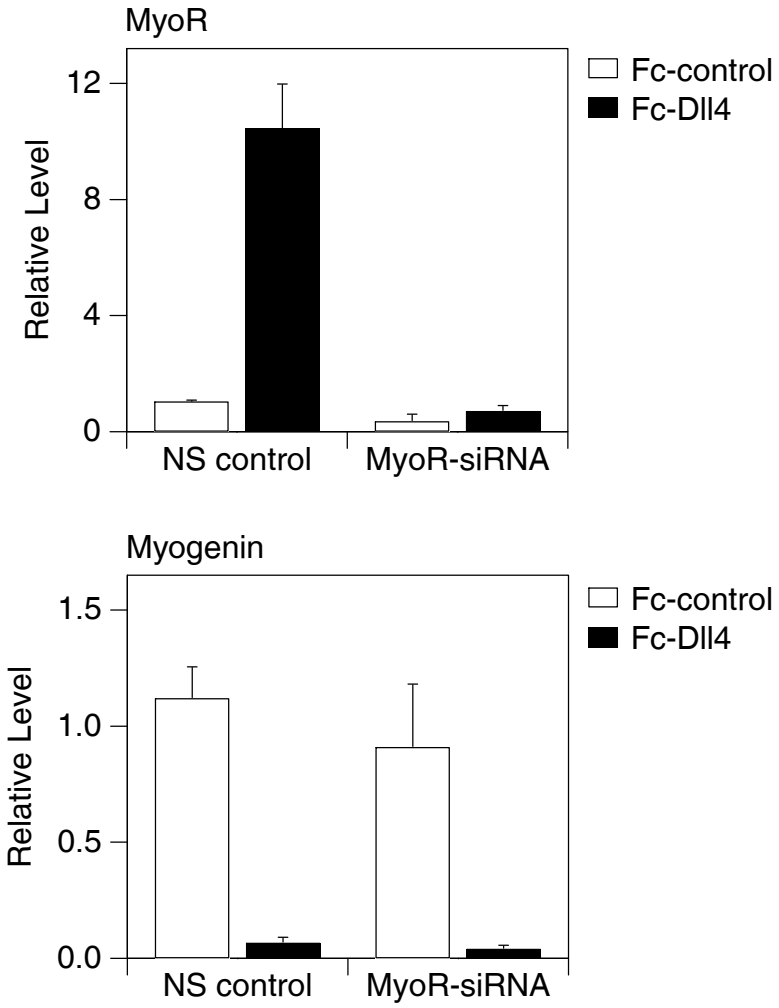

Figure 2.17. Ligand-induced Notch signaling effectively blocks Myogenin induction in cells expressing reduced levels of MyoR. Individual tissue culture wells were treated with Fc-Dll4 or Fc-control as indicated in the Methods. C2C12 cells transfected with either non-silencing (NS) control siRNA oligonucleotides or MyoR-directed siRNAs were propagated on the coated plates and then shifted to DM for 24 hours. Expression of MyoR (upper panel) and Myogenin (lower panel) RNA was assessed by quantitative RT-PCR. Q-PCR values are presented as the average +/- standard deviation of three replicate samples.

to obtain robust induction of MyoR, a less sensitive Notch-responsive gene than Hey1. The reduced level of MyoR, however, did not compromise the ability of Notch to repress the induction of Myogenin (Figure 2.17, bottom), or of two additional markers, Mef2C and Myh3 (Figure 2.18). This result is consistent with our previous data indicating that Hey1 appears sufficient to account for the effects of Notch on early myogenesis; in the absence of MyoR, Hey1 would be expected to compensate.

To investigate the existence of any additional MyoR- and Hey1-independent pathways, I employed siRNAs to simultaneously knock-down both Hey1 and MyoR expression. Transfection of C2C12 cells with this mixture of siRNAs resulted in >85 percent reduction of both Hey1 and MyoR RNA levels when cultures were plated on Fc-Dll4 (Figure 2.19, top). Despite the drastically reduced levels of Hey1 and MyoR, exposure to Fc-Dll4 still effectively repressed induction of Myogenin at 24 hours in DM (Figure 2.19, bottom). Similar results were obtained when cultures were taken out to three days in DM and analyzed for Myh3 induction (Figure 2.20), with the caveat that knock-down efficiency had declined to ~65 percent. Myoblast fusion at four days in DM also continued to be repressed in cultures treated with Hey1/MyoR siRNAs and plated on Fc-Dll4 (Figure 2.21). While I cannot rule out the possibility that low levels of residual Hey1 or MyoR are sufficient to block myogenesis, our data suggest that Notch signaling inhibits myogenesis through multiple pathways, and that yet additional mediators beyond Hey1 and MyoR are likely to contribute to the pathway's biological effects in our system.

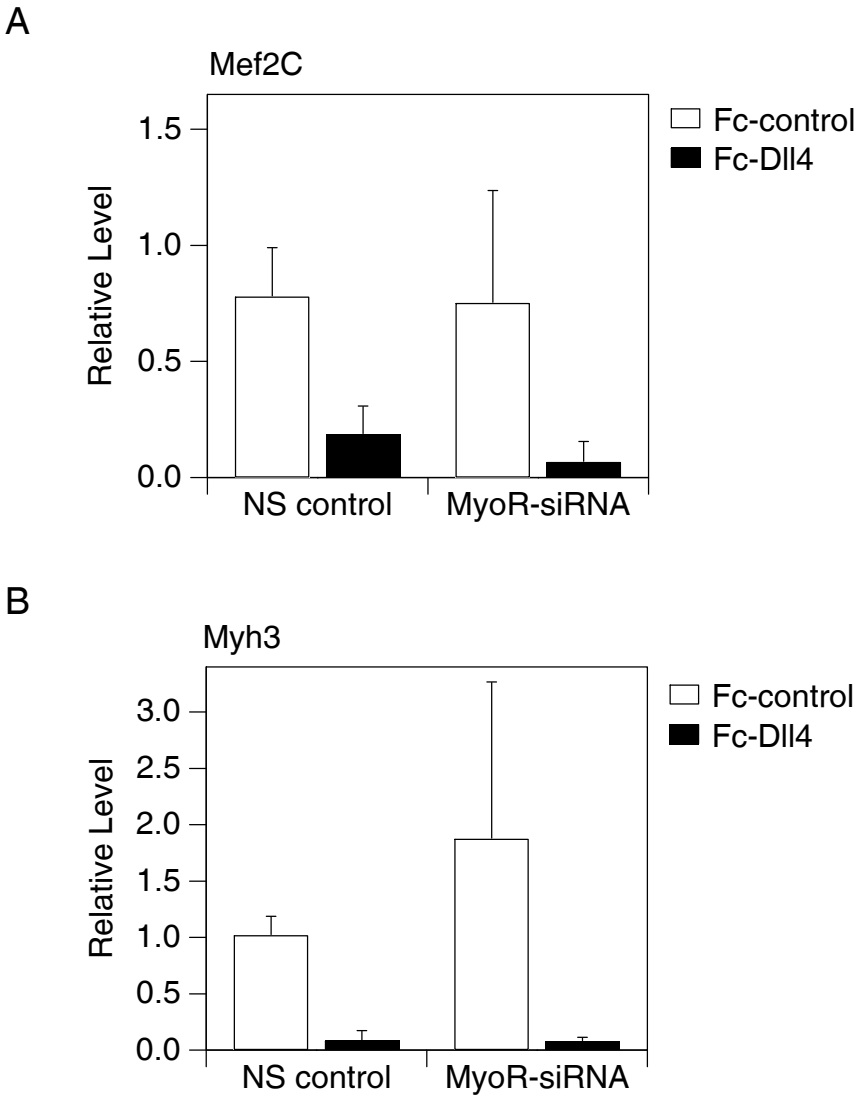

Figure 2.18. Ligand-induced Notch signaling effectively blocks induction of Mef2C and Myh3 in cells expressing reduced levels of MyoR. Individual tissue culture wells were treated with Fc-Dll4 or Fc-control as indicated in the Methods. C2C12 cells transfected with either non-silencing (NS) control siRNA oligonucleotides or MyoR-directed siRNAs were propagated on the coated plates and then shifted to DM for 24 hours. Expression of (A) Mef2C and (B) Myh3 RNA was assessed by quantitative RT-PCR. Q-PCR values are presented as the average +/- standard deviation of three replicate samples. Note: While MHC protein levels were only appreciably induced in control cells by 48 hours in DM, Myh3 RNA was elevated ~300 fold by 24 hours (data not shown).

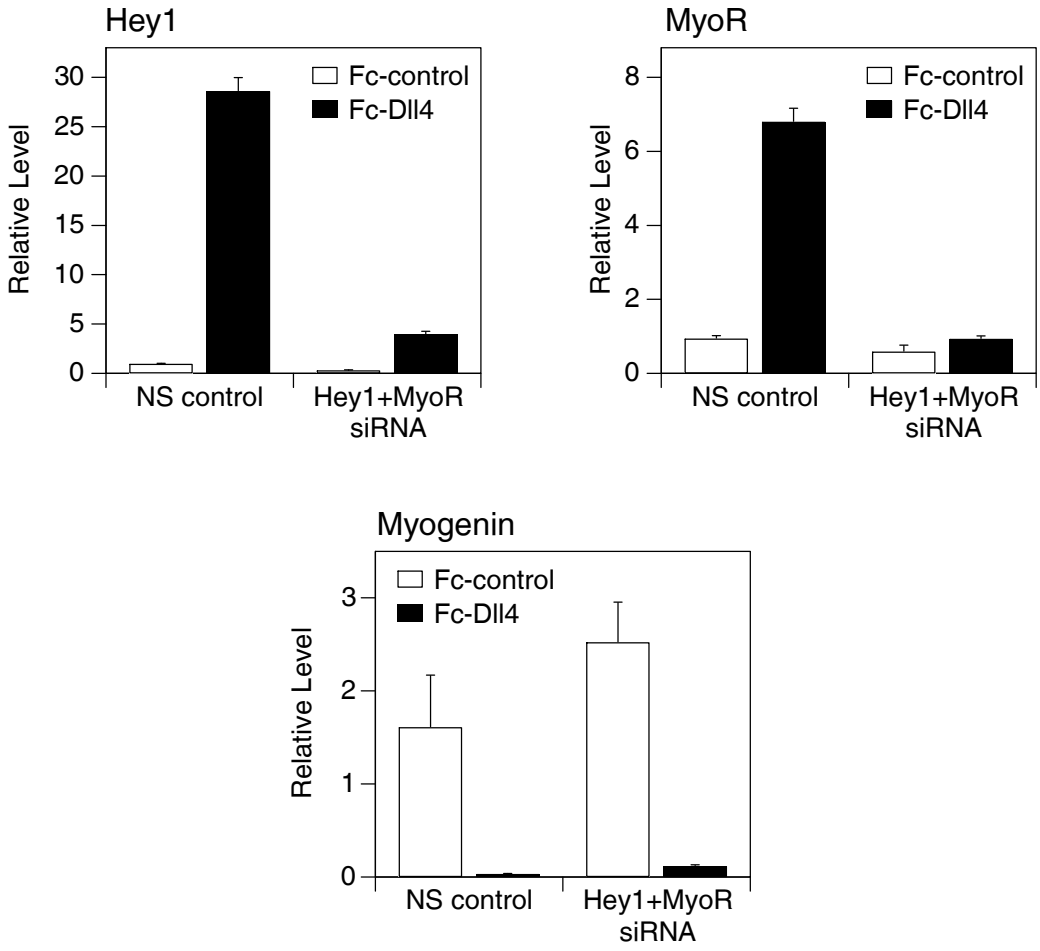

Figure 2.19. Ligand-induced Notch signaling effectively blocks Myogenin induction in cells expressing reduced levels of Hey1 and MyoR. Individual tissue culture wells were treated with Fc-Dll4 or Fc-control as indicated in the Methods. C2C12 cells transfected with either non-silencing (NS) control siRNA oligonucleotides or a mixture of Hey1-directed and MyoR-directed siRNAs were propagated on the coated plates and then shifted to DM for 24 hours. Expression of Hey1 (upper left panel), MyoR (upper right panel), and Myogenin (lower panel) RNA was assessed by quantitative RT-PCR. Q-PCR values are presented as the average +/- standard deviation of three replicate samples.

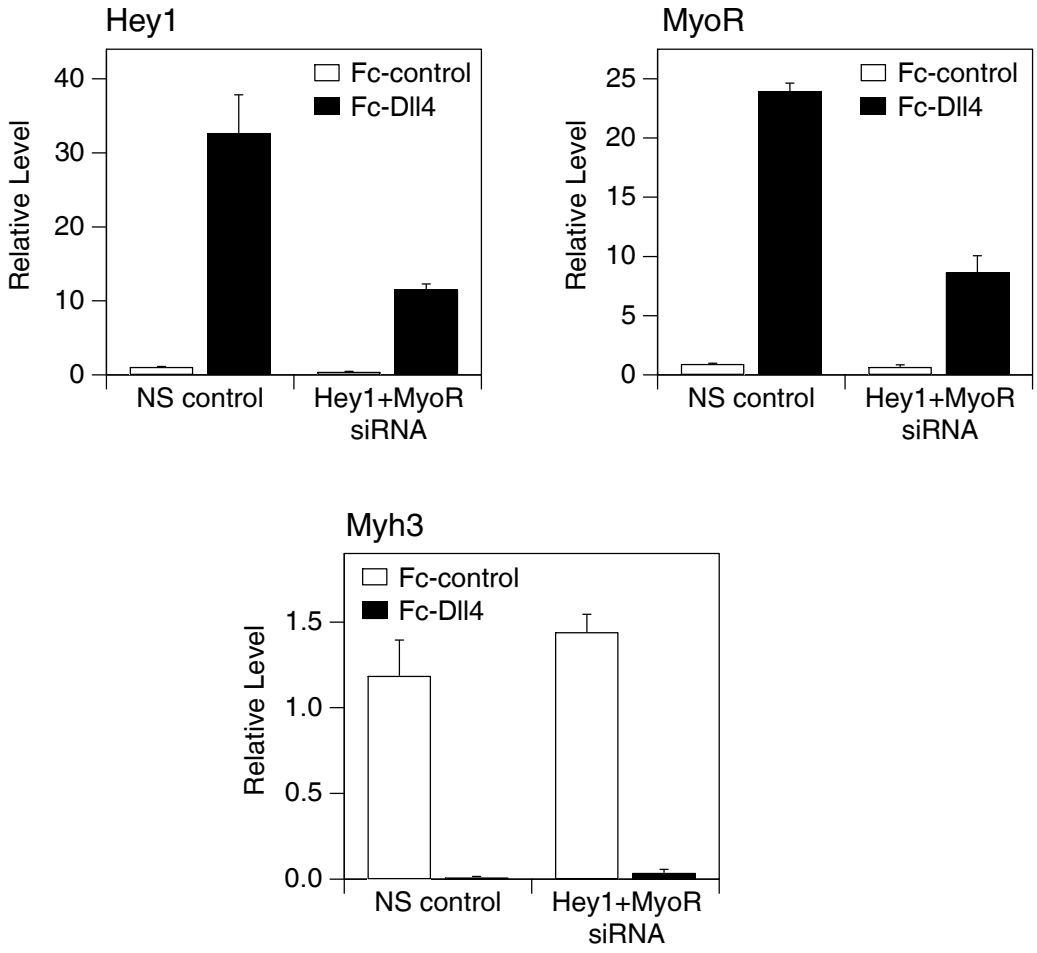

Figure 2.20. Ligand-induced Notch signaling effectively blocks Myh3 induction in cells expressing reduced levels of Hey1 and MyoR. Individual tissue culture wells were treated with Fc-Dll4 or Fc-control as indicated in the Methods. C2C12 cells transfected with either non-silencing (NS) control siRNA oligonucleotides or a mixture of Hey1-directed and MyoR-directed siRNAs were propagated on the coated plates and then shifted to DM for 72 hours. Expression of Hey1 (upper left panel), MyoR (upper right panel), and Myh3 (lower panel) RNA was determined by quantitative RT-PCR. Q-PCR values are presented as the average +/- standard deviation of three replicate samples.

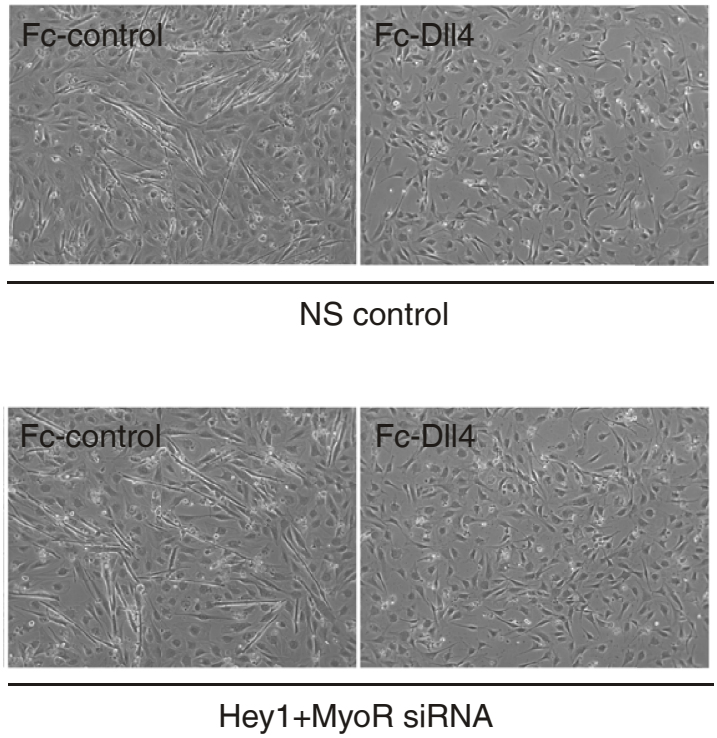

Figure 2.21. Ligand-induced Notch signaling effectively blocks myoblast fusion in cells expressing reduced levels of Hey1 and MyoR. Individual tissue culture wells were treated with Fc-Dll4 or Fc-control as indicated in the Methods. C2C12 cells were transfected with either non-silencing (NS) control siRNA oligonucleotides or a mixture of Hey1-directed and MyoR-directed siRNAs. Cultures were propagated on the coated plates in growth medium (GM), shifted to differentiation medium (DM) for four days, and assessed for myoblast fusion.

DISCUSSION

Notch signaling was shown over a decade ago to inhibit myogenesis in cultured cells and more recently to prevent the premature differentiation of muscle progenitor cells and satellite cells in-vivo (Conboy and Rando, 2002; Kopan et al., 1994; Lindsell et al., 1995; Schuster-Gossler et al., 2007; Vasyutina et al., 2007). However, the molecular pathways through which Notch exerts its inhibitory effects have not been clearly defined. My results provide evidence that Notch acts through multiple pathways to repress myogenesis. Notch induced the expression of a multitude of genes in cultured myoblasts, and individual constitutive expression of at least two of them, Hey1 and MyoR, was sufficient to block (or significantly delay) myogenesis. Consistent with a model in which no single factor downstream of Notch is required for myogenic inhibition, siRNA knockdown experiments directed against either Hey1 or MyoR revealed that significantly reducing the dosage of either of these factors had no appreciable effect on the ability of Notch to exert repression. Intriguingly, even simultaneous knockdown of both Hey1 and MyoR did not appear to rescue repression by Notch in any substantial fashion, suggesting the existence of additional contributory factors downstream of the pathway.

Notch affects myogenesis at an early step, inhibiting the induction of Myogenin and Mef2C. While MyoD expression levels were moderately reduced in cells exposed to Notch ligands, the extent of this down-regulation was not as severe as previously reported (Kuroda et al., 1999) and is unlikely to account for the complete block to myogenesis. Rather, it appears that Notch primarily functions to antagonize the ability of MyoD to activate downstream myogenic target genes. Since past work has shown that MyoD can

induce its own expression (Thayer et al., 1989), the observed reduction in MyoD RNA levels may reflect an indirect consequence of compromised MyoD transcriptional activity. Interestingly, Notch signaling led to a three-fold increase in Myf-5 transcripts. This induction could potentially reflect either the direct action of NICD on the Myf-5 promoter, or an indirect effect of reduced MyoD expression, as Myf-5 levels are also elevated in the MyoD knockout mouse (Rudnicki et al., 1992). Whether the skewed expression pattern of increased Myf-5 (and reduced MyoD) has any functional significance in our system remains an open question. At least one report has argued that Myf-5 may be inherently less potent than MyoD as a driver of the differentiation program in vitro (Ishibashi et al., 2005), although apparently compensates adequately for MyoD in vivo (Rudnicki et al., 1992). Elevated Myf-5 expression levels alone do not appear to exert a negative impact on myogenesis, as forced retroviral expression of Myf-5 in C2C12 cells was compatible with normal differentiation (data not shown).

Members of both the Hes and Hey (HRT, HERP, CHF) families of bHLH repressors can be induced by Notch. In agreement with others (Shawber et al., 1996), Hes1 was expressed in C2C12 cells, but poorly induced, and was not effective at blocking myogenesis (Shara Kabak, unpublished observation). Other Hes family members were not appreciably induced. By contrast, all three members of the Hey family, Hey1, Hey2 and HeyL, were induced by Fc-Dll4, but the overall level of Hey2 was very low. Constitutive expression of Hey1 repressed myogenesis while, surprisingly, HeyL had no effect. This argues that, despite a high level of structural similarity, the biological activities of the Hey proteins are distinct. Hey2, when expressed as a FLAG-tagged construct, did exhibit the ability to repress myogenesis (Figure 2.5); however,

given the very low level of endogenous Hey2 transcript induced by Fc-Dll4, I do not consider Hey2 to be a significant player in the mediating the effects of Notch in this system. My results do, however, implicate Hey1 as a potentially important Notch effector in myoblasts. My data showing that a reduced level of Hey1 has no effect on the response of cells to Notch were therefore unexpected. This was true even at levels of Notch signaling that only partially induced Hey1 and partially restricted Myogenin induction.

In contrast to a simple model of Notch acting primarily to induce the transcription of Hes or Hey family members, my expression screen revealed over 82 transcripts that were upregulated after only six hours of ligand stimulation. Several strongly induced genes did not inhibit myogenesis when tested functionally by constitutive expression (Nrarp, Trib2, G22). However, whether or not these genes play any functional role in muscle remains to be determined. For example, it is possible that these genes exhibit little impact when expressed individually, but will affect myogenesis when expressed in combination. Despite this possibility, my screen suggests that Notch may generally induce a large number of genes, but employ only specific subsets of these to execute the pathway's effects in different cell types.

Importantly, my work has identified MyoR as a novel Notch-responsive gene that appears to contribute to myogenic repression. MyoR was originally identified in a screen for cDNAs with homology to capsulin, another bHLH transcription factor (Lu et al., 1999). MyoR exhibits a skeletal muscle-specific pattern of embryonic expression and has been shown to antagonize the activity of MyoD in reporter assays and bind to E-box DNA elements in vitro. Accordingly, it was suggested that this bHLH inhibitor functions to delay the progression of myogenesis during development. MyoR was also found to be

induced during muscle regeneration, arguing for an additional role in satellite cells (Zhao and Hoffman, 2006). ABF-1, the putative human orthologue of MyoR, was cloned concurrently from activated B-cells (Massari et al., 1998), suggesting muscle-independent functions.

My data are consistent with a model in which Notch signaling acts through at least two myogenic inhibitors, Hey1 and MyoR, to repress myoblast differentiation in culture. It is tempting to speculate that these same proteins may also represent important arms of the Notch pathway during embryonic and/or post-natal myogenesis in-vivo. Impairment of Notch activity results in premature progenitor cell differentiation in the embryo and compromised satellite cell proliferation in the adult (Conboy et al., 2003; Schuster-Gossler et al., 2007; Vasyutina et al., 2007). While knockout mice deficient for either Hey1 or MyoR have been generated and do not exhibit overt embryonic muscle phenotypes (Fischer et al., 2004; Lu et al., 2002), the absence of such defects in single-knockout animals is consistent with a model in which multiple factors downstream of Notch contribute to the pathway's phenotypic effects. It has been observed that Hey1 and MyoR collaborate with related transcription factors, Hey2 and Capsulin, respectively. Hey1 and Hey2 act redundantly in the embryonic vasculature (Fischer et al., 2004), while MyoR and Capsulin function redundantly in the formation of the facial musculature (Lu et al., 2002). Preliminary in-situ hybridization studies have revealed a striking overlap in the expression patterns of Hey1 and MyoR in E10.5 embryos (Figure 2.22), a finding consistent with both distinct and coordinate regulation of these two genes.

In light of the results from my double knockdown experiment (Figures 2.19-2.21), it appears likely that additional factors beyond Hey1 and MyoR are important in

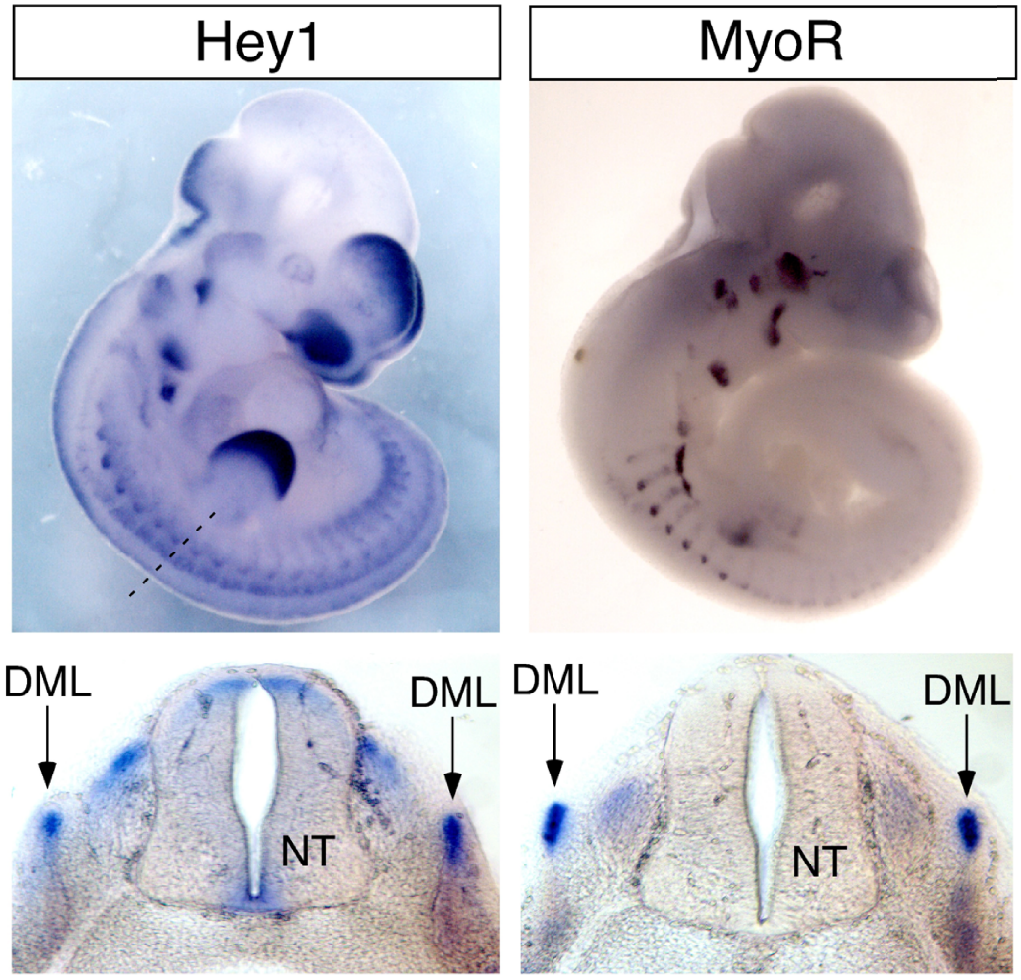

Figure 2.22. RNA expression patterns of Hey1 and MyoR. E10.5 day embryos (upper panels) and transverse sections (lower panels) were analyzed by in situ hybridization using probes specific for Hey1 or MyoR. The dashed line indicates the approximate position of the sections shown. NT: neural tube. DML: dorsal medial lip.
Data courtesy of Dr. Doug Epstein.

contributing to Notch-mediated inhibition of myogenesis. The large number of Notch-responsive genes identified by my array is consistent with the notion that Notch activates an extensive gene network in order to execute its critical functions in muscle. My follow-up studies have shown that at least two additional Notch-responsive genes, when constitutively expressed, can recapitulate the block to C2C12 differentiation. First, consistent with a previous report (Atherton et al., 1996), I demonstrated that forced expression of the HLH protein Id3 inhibited myogenesis (Figure 2.23). Id proteins, which lack a basic domain and cannot bind DNA, are thought to function by forming inactive heterodimers with E proteins or MyoD (Benezra et al., 1990; Jen et al., 1992). Notch activity has also been linked to Id3 induction in Xenopus (Reynaud-Deonauth et al., 2002). Second, I later showed that another gene identified by the array, the transcription factor GATA3, was indeed induced by Notch in C2C12 cells (Figure 2.24) and also repressed differentiation (Figures 2.25 & 2.26). The work of others revealed that Notch can induce GATA3 in T cells (Amsen et al., 2007; Fang et al., 2007), but an inhibitory role for this protein in myogenesis has not been previously reported. Interestingly, GATA3 binding sites are present within the Myogenin and Mef2C promoters, but the mechanism by which this factor represses myogenic transcription remains to be determined. While the levels of Id3 and GATA3 induction observed in our system in response to Fc-Dll4 are relatively modest, these factors, in combination with other Notch-responsive genes, such as IL-6, or even the negligible level of Hey2 induced by ligand stimulation, may indeed participate in myogenic repression.

The positioning of activated Notch as the hub of a transcriptional network containing multiple effectors, many of which may contribute functionally to the

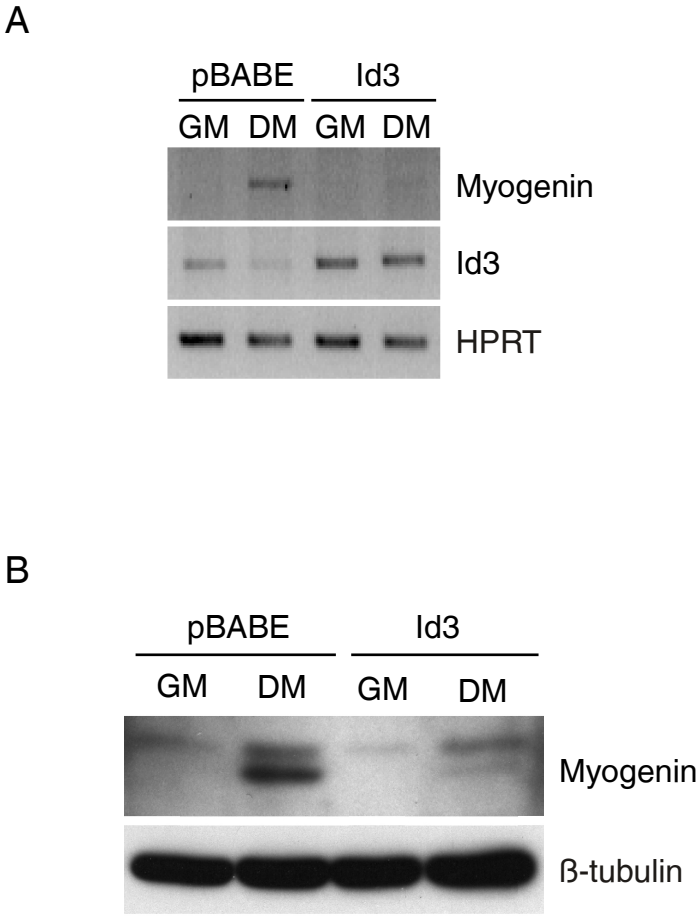

Figure 2.23. Constitutive expression of Id3 inhibits myogenesis. C2C12 cells were stably transduced with parental retrovirus (pBABE-puro) or a retrovirus expressing HA-tagged Id3. (A) Lines were propagated in growth medium (GM) and then shifted to differentiation medium (DM) for 24 hours and analyzed for expression of Myogenin and Id3 by RT-PCR using HPRT as a loading control. (B) Lines were propagated in GM, shifted to DM for 24 hours and analyzed for expression of Myogenin by Western immunoblotting using ß-tubulin as a loading control.

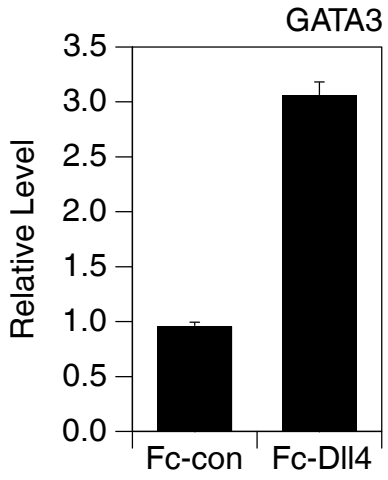

Figure 2.24. GATA3 is induced by ligand-mediated Notch signaling in myoblasts. 10 cm dishes were coated with 2.5 ml of ligand-containing supernatant. C2C12 myoblasts were plated on either Fc-Dll4 or Fc-control ligand and propagated in growth medium (GM) for six hours. GATA3 RNA levels were determined by quantitative RT-PCR using 18S as a loading control. Data represent averages +/- standard deviation of three replicate samples.

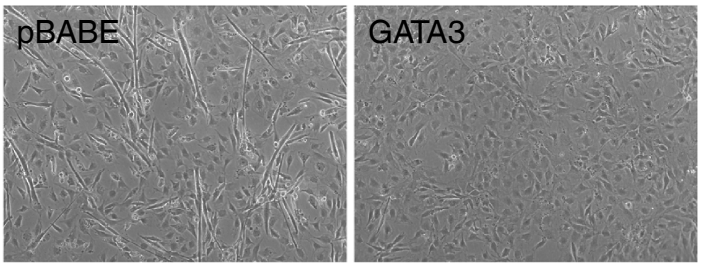

Figure 2.25. Constitutive expression of GATA3 inhibits myoblast fusion. C2C12 cells were stably transduced with parental retrovirus (pBABE-puro) or a retrovirus expressing FLAG-tagged GATA3. Fusion of myoblasts into myotubes was examined in the indicated cell lines after three days in DM.

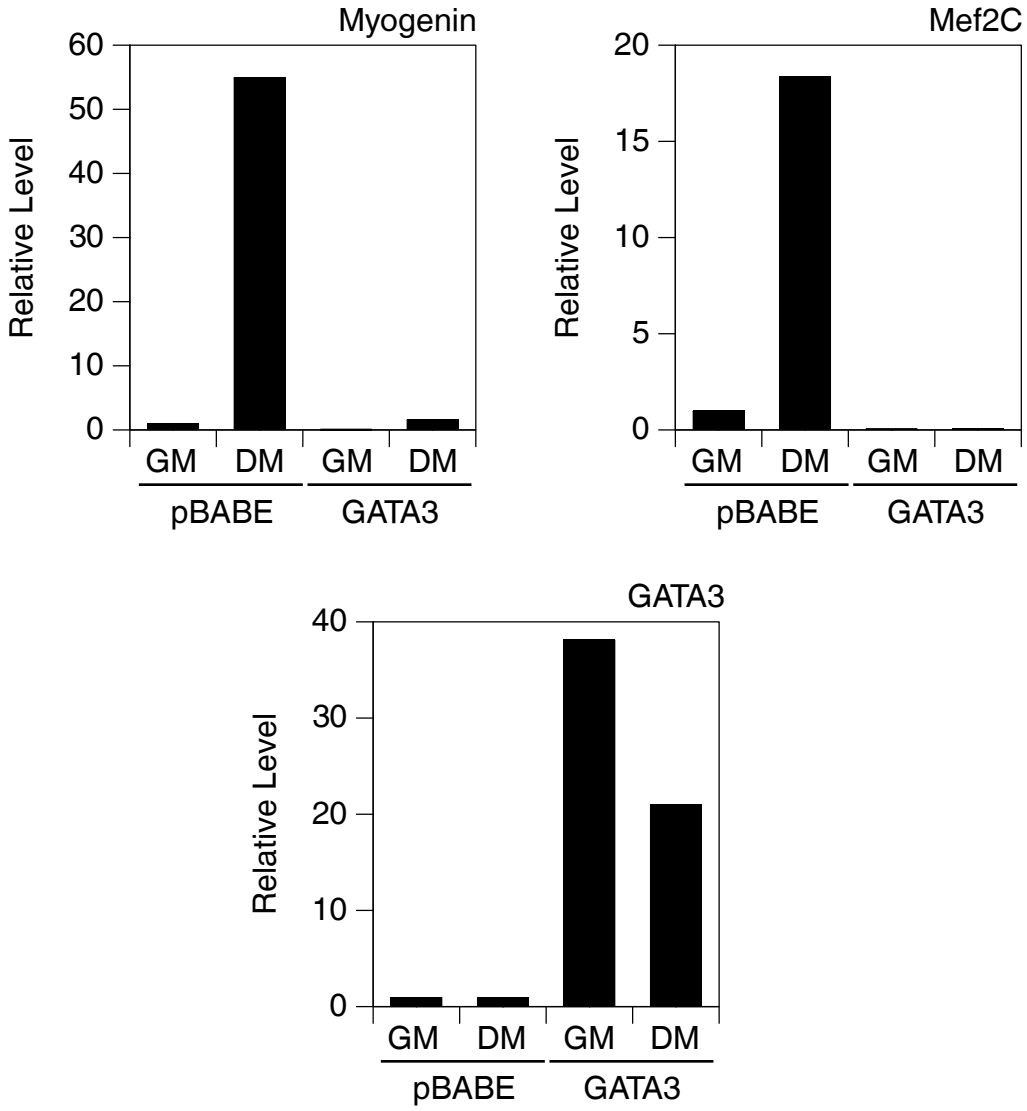

Figure 2.26. Constitutive expression of GATA3 inhibits myogenesis. C2C12 cells were stably transduced with parental retrovirus (pBABE-puro) or a retrovirus expressing FLAG-tagged GATA3. Lines were propagated in growth medium (GM) and then shifted to differentiation medium (DM) for 24 hours and analyzed for expression of Myogenin (upper left panel), Mef2C (upper right panel), and GATA3 (lower panel) by quantitative RT-PCR using 18S as a loading control.

pathway's biological effects, is likely to be applicable to tissues other than muscle. Generally, such a framework may serve to render any one particular target of a signaling cascade dispensable for the overall phenotypic consequences of the pathway. In the mammalian nervous system, it appears that the transcriptional repressors Hes1 and Hes5 represent the primary mediators of the pathway's inhibitory effects on differentiation (Ohtsuka et al., 1999). By contrast, in the epidermis, Notch acts directly through both Hes1 and the cell cycle inhibitor p21 (Mammucari et al., 2005; Rangarajan et al., 2001). The specific targets (nodes) employed by Notch may differ from tissue to tissue, but the principle of functional redundancy could represent a general feature that ensures a robust signaling response. Redundancy and associated robustness are critical attributes of complex physiological systems that enhance their capacity to evolve (Kirschner and Gerhart, 1998).

Chapter III. The Notch Effector Hey1 Associates with Myogenic Target Genes to Repress Myogenesis

ABSTRACT

Members of the Hey family of transcriptional repressors are basic helix-loop-helix proteins that are thought to act downstream of Notch in diverse tissues. While forced expression of Hey1, a target of Notch in myoblasts, is sufficient to recapitulate the pathway's inhibitory effects on differentiation, how Hey1 interferes with myogenic transcription has not been fully elucidated. I provide multiple lines of evidence that Hey1 does not target the intrinsic transcriptional activity of the skeletal muscle master regulator MyoD. My results indicate instead that Hey1 is recruited to the promoter regions of Myogenin and Mef2C, two genes whose induction is critical for myogenesis. Expression of Hey1 in C2C12 myoblasts correlates with reduced recruitment of MyoD to these promoters, arguing that Hey1 inhibits myogenesis by associating with and repressing expression of key myogenic targets.

RESULTS

My previous work suggested that ligand-mediated Notch signaling acts through multiple pathways to repress myogenesis (Chapter II). I next sought to determine how individual Notch effectors inhibit the myogenic transcriptional program. I focused my initial analysis on Hey1, as the molecular mechanisms by which Hey proteins function in specific biological contexts are not well understood. While induction of Hey1 was not required for Notch-mediated inhibition, this finding likely reflects the fact that Hey1 functions together or in parallel with other repressors (eg. MyoR, Id3, or GATA3) to mediate the effects of Notch. In systems where functional redundancy may be a defining feature, it is only by unraveling the modes of action of individual effectors that we can reach a complete understanding of the pathway as a whole.

Hey1 does not repress intrinsic MyoD transcriptional activity

Past studies have demonstrated that Hey1 inhibits MyoD-mediated myogenic conversion of cultured fibroblasts (Sun et al., 2001) and the differentiation of C2C12 myoblasts (Chapter II). An important question relates to whether this inhibition reflects a generalized block to MyoD-driven transcription, or rather a selective targeting of individual myogenic promoters. To address this, I performed transfection assays in 10T1/2 fibroblasts. When these cells are transfected with a vector expressing MyoD, they convert to a muscle phenotype and express a wide array of myogenic transcripts. I reasoned that if Hey1 were repressing the inherent ability of MyoD to activate gene expression, the induction of all targets downstream of MyoD should be compromised in

the presence of Hey1. Two of the earliest markers activated in this system are the cell adhesion molecule Cadherin-15 and the muscle regulatory factor Myogenin. While transfection of 10T1/2 cells with a MyoD-expression plasmid robustly induced both of these genes, as expected, co-expression of Hey1 inhibited the induction only of Myogenin, and not Cadherin-15 (Figure 3.1). This apparent specificity in Hey1-mediated repression was also observed in luciferase reporter assays. Two reporter constructs were employed, one consisting of the proximal 133-bp Myogenin promoter (G133-luciferase), and the other composed simply of four high-affinity MyoD consensus E-box elements linked to a minimal promoter (4RE-tk-luciferase). 4RE-tk-luciferase has been used previously as a readout of "pure" MyoD activity, as this construct does not contain binding sites for any MyoD cofactors, such as Mef2 (Lu et al., 2000). While MyoD robustly induced both reporters, Hey1 repressed the induction only of G133-luciferase, and not 4RE-tk-luciferase (Figure 3.2). Together, these results strongly suggest that Hey1's inhibitory effects on myogenesis reflect promoter-specific repression of select myogenic targets, rather than generalized inhibition of MyoD activity.

Hey1 does not form dimers with MyoD or E47 or disrupt endogenous MyoD:E47 complexes

While the above functional data revealed that not all MyoD-responsive promoters are subject to repression by Hey1, a past report had proposed that Hey1 targets MyoD itself, by sequestering it into inactive heterodimers (Sun et al., 2001). To revisit this proposal, I evaluated whether Hey1 associates with MyoD or its binding partner E47 to repress myogenic transcription. I first performed co-immunoprecipitation assays in which

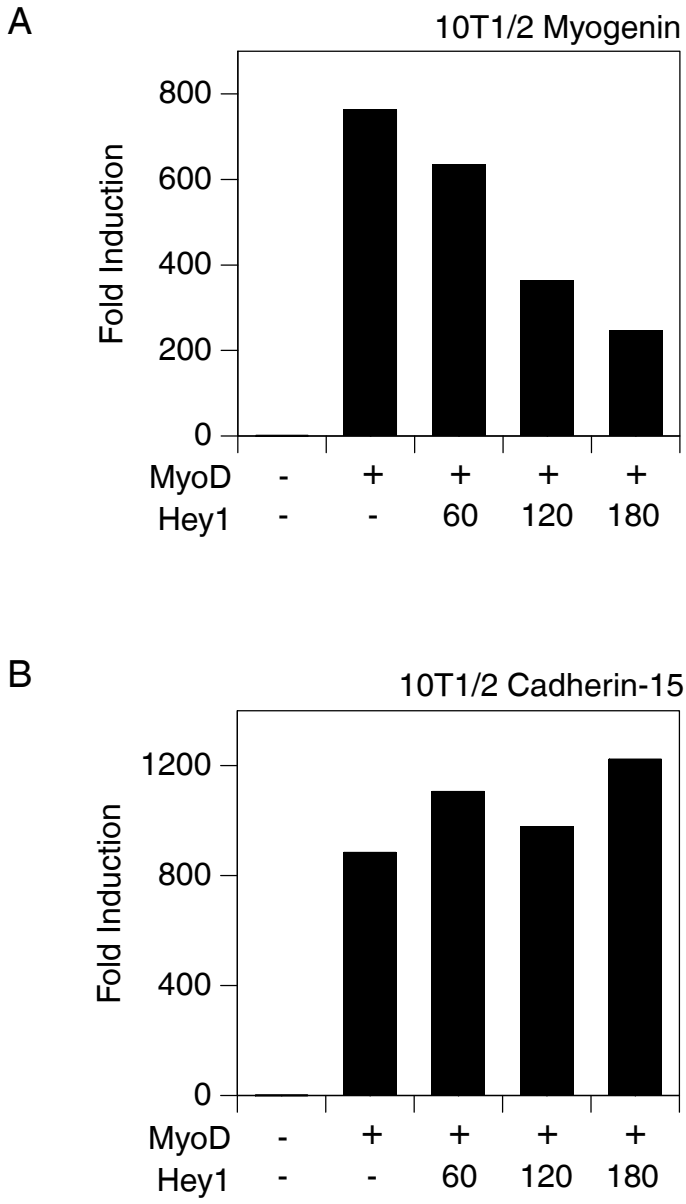

Figure 3.1. Hey1 exerts promoter-specific repression of myogenesis. 10T1/2 cells were transfected with 30 ng of pEMSV-MyoD alone or in combination with increasing amounts of pcDNA3.1-Hey1-V5 (60-180 ng). Myogenin (A) and Cadherin-15 (B) RNA levels were determined by quantitative RT-PCR.

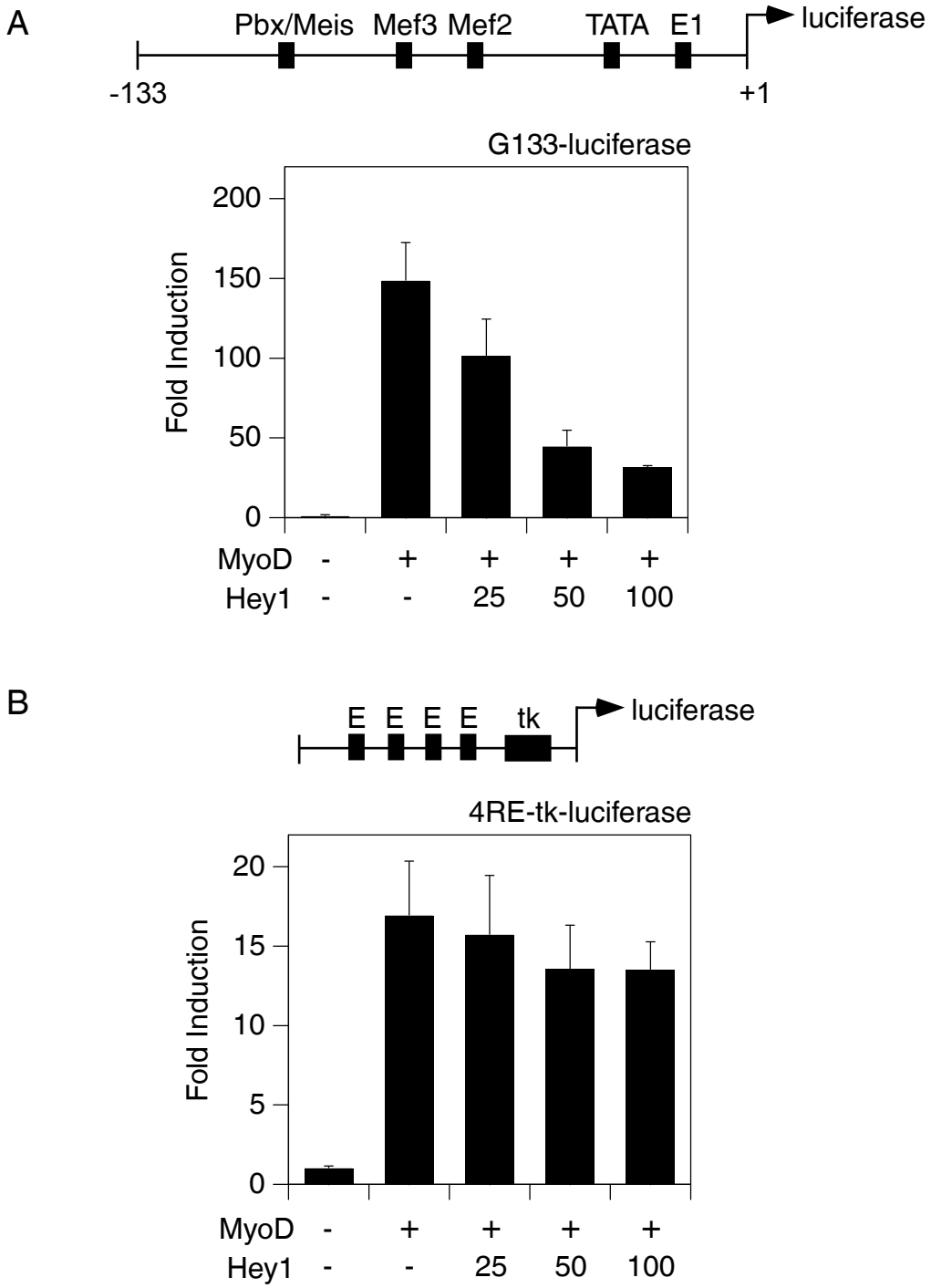

Figure 3.2. Hey1 represses the induction of G133-luciferase but not 4RE-tk-luciferase. 10T1/2 cells were transfected with 25 ng pRL-TK-Renilla, 25 ng G133-luciferase (A) or 25 ng 4RE-tk-luciferase (B), 25 ng pEMSV-MyoD, and 25-100 ng of pcDNA3.1-Hey1-V5. Firefly luciferase values were normalized to Renilla luciferase and plotted as averages of three replicate samples +/- standard deviation.

I overexpressed either MyoD in combination with E47 or Hey1 (Figure 3.3), or E47 in combination with MyoD or Hey1 (Figure 3.4). 293T cells were used for these experiments, as they allow for very high transfection efficiencies and levels of expressed proteins, raising the likelihood that even weak interactions can be detected. These studies confirmed the expected binding of MyoD to E47 (Figure 3.3, lane 5; Figure 3.4, lane 5) but failed to reveal any evidence of association between either of these factors and Hey1 (Figure 3.3, lane 6; Figure 3.4, lane 6). The ability of Hey1 to dimerize with itself was verified independently using the Hey1 construct employed in these assays in combination with a Myc-tagged Hey1 (data not shown).

In parallel, I used electrophoretic mobility shift assays (EMSAs) to determine whether forced expression of Hey1 in C2C12 myoblasts would disrupt the formation of endogenous MyoD:E47 complexes. Nuclear extracts were prepared from C2C12 cells stably transduced with either the pBABE-puro or pBABE-FLAG-Hey1 retrovirus. As a control, I examined extracts of control 293T cells or 293T cells co-transfected with MyoD and E47 expression plasmids. When these 293T extracts were incubated with a radio-labeled probe containing a high-affinity E-box, complexes corresponding to MyoD:E47 heterodimers were readily observed (Figure 3.5A, compare lanes 2 and 3). A MyoD antibody shifted the mobility of the complexes, confirming that they contain MyoD (lane 4). Similar complexes were observed in the C2C12 cell extracts (Figure 3.5A, lanes 5-8). Importantly, these complexes were not appreciably affected by the presence of Hey1 (compare lanes 5 and 6 to lanes 7 and 8). The complex migrating just below what I tentatively identify as MyoD homodimers in extracts from the pBABE-transduced cells was not shifted by a MyoD antibody, but was shifted with a Myogenin

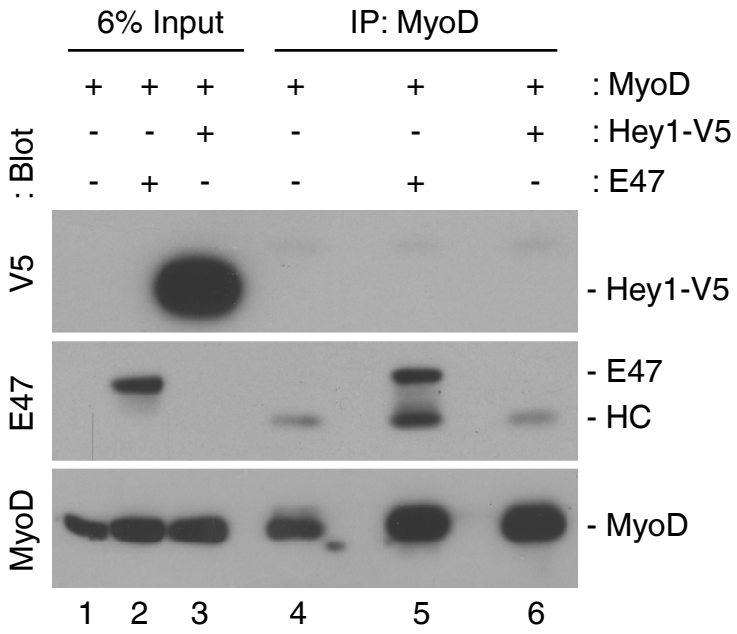

Figure 3.3. Hey1 does not form heterodimers with MyoD. 293T cells were transfected with 2 µg of pEMSV-MyoD alone or in combination with 2 µg CMV-E47 or 2 µg pcDNA-3.1-TOPO-Hey1-V5. Lysates were harvested after 48 hours and subjected to immunoprecipitation with MyoD-specific antibodies. Immunoprecipitates and input samples were immuno-blotted with anti-V5, anti-E47, or anti-MyoD antibodies. HC, heavy chain.

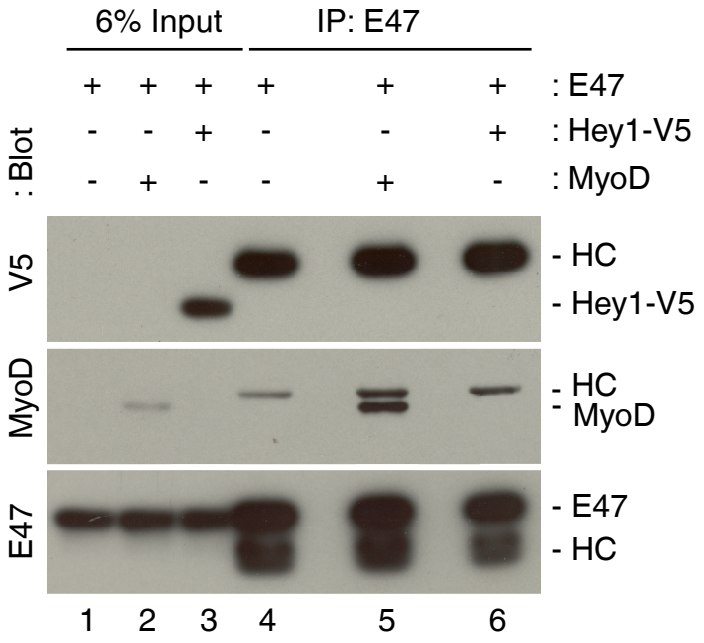

Figure 3.4. Hey1 does not form heterodimers with E47. 293T cells were transfected with 2 µg of CMV-E47 alone or in combination with 2 µg pEMSV-MyoD or 2 µg pcDNA-3.1-TOPO-Hey1-V5. Lysates were harvested after 48 hours and subjected to immunoprecipitation with E47-specific antibodies. Immunoprecipitates and input samples were immuno-blotted with anti-V5, anti-MyoD, or anti-E47 antibodies. HC, heavy chain.

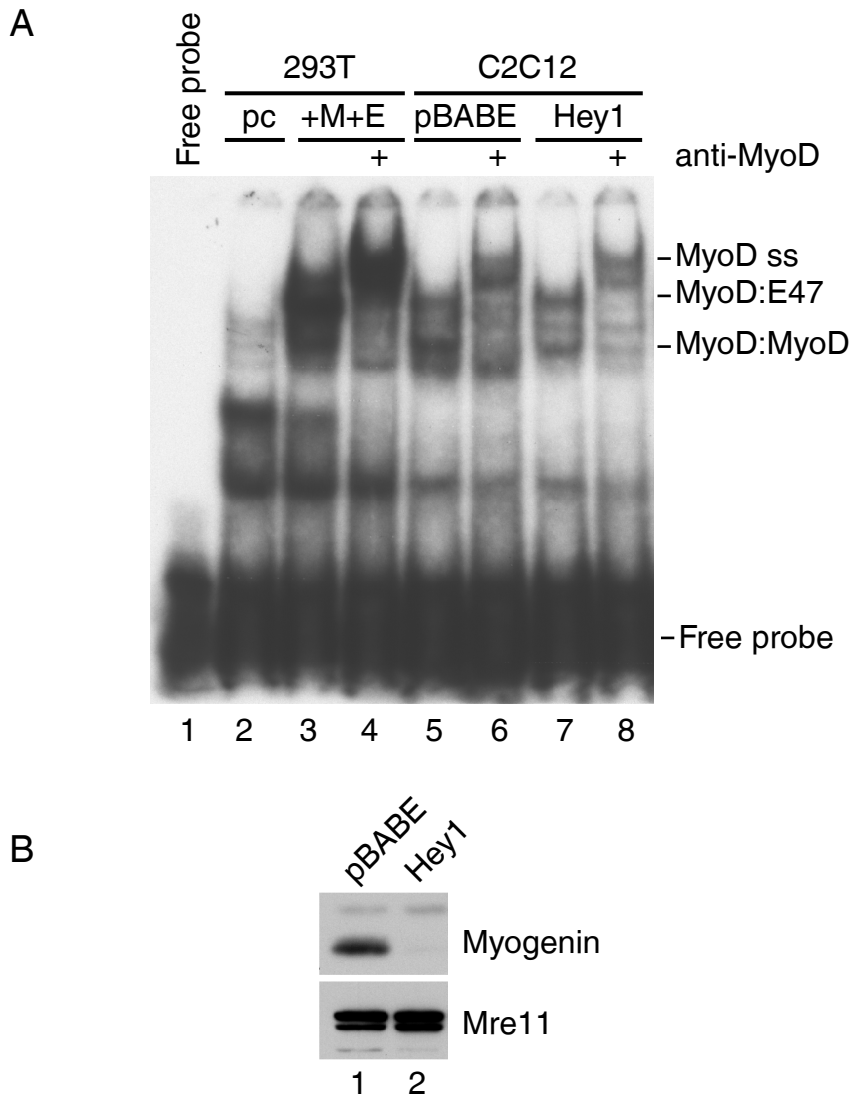

Figure 3.5. Hey1 does not disrupt endogenous MyoD:E47 complexes in C2C12 cells. (A) Nuclear extracts were harvested from C2C12 cells stably transduced with pBABE-puro or pBABE-FLAG-Hey1 and maintained in differentiation medium (DM) for 24 hours. Nuclear extracts were also isolated from 293T cells transiently transfected with either 6 μg pcDNA3.1 empty vector or 2 μg pEMSV-MyoD, 2 μg CMV-E47, and 2 μg pcDNA3.1. Extracts were incubated with a 30-nucleotide ^{32}P-labeled probe containing the high-affinity MyoD E-box (CAGGTG) found within the Mef2C promoter and MCK enhancer. Protein-DNA complexes were incubated with or without MyoD-specific antibodies prior to resolution via non-denaturing SDS-PAGE. Supershifted MyoD-containing complexes are indicated "MyoD ss". (B) Nuclear extracts from pBABE or pBABE-Hey1 C2C12 cells used in (A) were Western blotted with antibodies specific for Myogenin or Mre11 as a loading control.

antibody (data not shown). As expected, this complex was not observed in extracts from Hey1-expressing cells, consistent with the lack of Myogenin protein (Figure 3.5B). Taken together, our results indicate that Hey1 does not disrupt MyoD:E47 heterodimers, a finding in agreement with our initial functional data demonstrating promoter-specific repression by Hey1.

A role for Mef2C inhibition in Hey1-mediated repression of myogenesis

Our previous results (Figures 3.1 & 3.2) showed that Hey1 inhibits MyoD-mediated induction of the endogenous Myogenin gene and a transfected Myogenin promoter, but not the endogenous Cadherin-15 gene or a transfected E-box-driven reporter. I sought to investigate the basis for this specificity. Past work in cultured fibroblasts by Tapscott and colleagues demonstrated that Cadherin-15, but not Myogenin, is transcriptionally induced by MyoD in the absence of new protein synthesis (Bergstrom et al., 2002). This finding suggested that MyoD must collaborate with a secondary mediator or coactivator to activate Myogenin transcription. Indeed, other studies revealed that Myogenin induction, which is absolutely required for myogenesis in vivo, requires both MyoD and Mef2 proteins (Edmondson et al., 1992; Hasty et al., 1993; Nabeshima et al., 1993). Mef2C is itself a target of MyoD (Dodou et al., 2003; Wang et al., 2001) and has been shown to be the only Mef2 family member transcriptionally upregulated upon differentiation in C2C12 cells (Figure 2.1B). I considered the possibility that Hey1 represses myogenesis primarily by repressing Mef2C activity and/or Mef2C gene transcription, not the Myogenin promoter per se. To explore this possibility, I first confirmed that Mef2 is critical for Myogenin promoter activity. Indeed, mutation of the

single Mef2 element within the Myogenin proximal promoter dramatically reduced MyoD-stimulated reporter activity (Figure 3.6). I next asked if Hey1 affects Mef2C activity. I transfected 10T1/2 cells with a Mef2C expression vector along with a reporter consisting of three Mef2 DNA binding elements upstream of a minimal promoter driving luciferase. Induction of this reporter by Mef2C was unaffected by increasing amounts of Hey1, indicating that Hey1 does not inhibit Mef2C transcriptional activity per se (Figure 3.7A). By contrast, transcriptional induction of the endogenous Mef2C gene by MyoD was significantly repressed by Hey1 (Figure 3.7B), a result consistent with those obtained with Hey1-transduced C1C12 cells (Figure 2.4A).

If Hey1 functions primarily by repressing Mef2C expression, then one would expect exogenous Mef2C to rescue Hey1-mediated repression of Myogenin. Reporter assays revealed that repression of the Myogenin promoter was reduced, but not eliminated by a Mef2C expression plasmid (Figure 3.8). These data suggest that inhibition of Mef2C expression likely contributes to Hey1-mediated repression of myogenesis, but that Hey1 may also function through additional inhibitory mechanisms.

Evaluation of in-vitro DNA binding by Hey1 to myogenic promoter elements

I next asked if Hey1 inhibits transcription by binding DNA within target gene promoters. As a first step, I determined the ability of Hey1 to bind elements within the Myogenin and Mef2C proximal promoters in vitro. Prior studies employing SELEX approaches derived an optimum binding site for Hey1, which is the E-box CACGTG. Closely related variants of this sequence bind Hey1 less well (Fischer et al., 2002; Iso et al., 2001b; Pichon et al., 2004). The 133-bp Myogenin promoter contains a single E-box

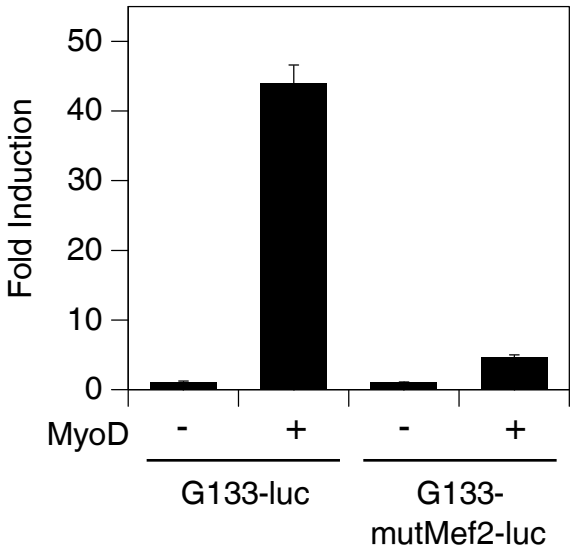

Figure 3.6. MyoD-mediated induction of the Myogenin minimal promoter requires an intact Mef2 element. 10T1/2 cells were transfected with 25 ng pRL-TK-Renilla, 25 ng G133-luciferase or G133-mutMef2-luciferase, and 25 ng pEMSV-MyoD. Firefly luciferase values were normalized to Renilla luciferase and plotted as averages of three replicate samples +/- standard deviation.

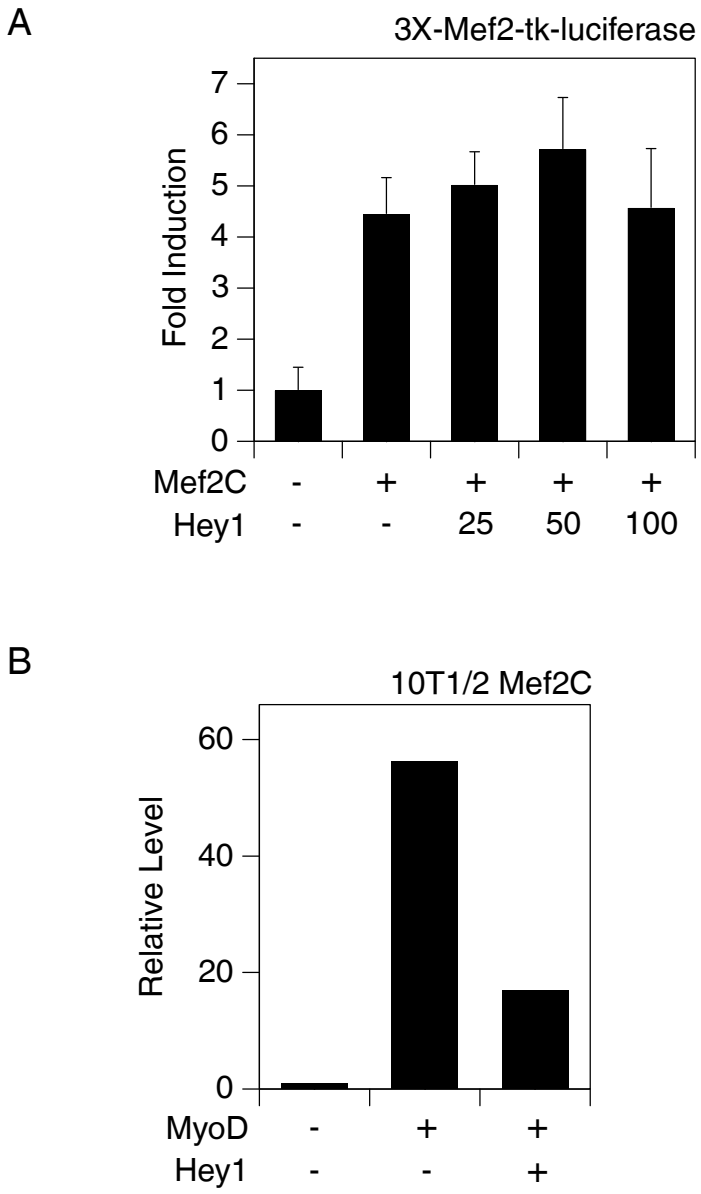

Figure 3.7. Hey1 inhibits the expression, but not the activity, of Mef2C. (A) 10T1/2 cells were transfected with 25 ng pRL-TK-Renilla, 25 ng 3X-Mef2-tk-luciferase, 25 ng pcDNA3.1-Mef2C (α1β), and 25-100 ng of pcDNA3.1-Hey1-V5. Firefly luciferase values were normalized to Renilla luciferase and plotted as averages of three replicate samples +/- standard deviation. (B) 10T1/2 cells were transfected with 30 ng of pEMSV-MyoD alone or in combination with 180 ng pcDNA3.1-Hey1-V5. Mef2C RNA levels were determined by quantitative RT-PCR.

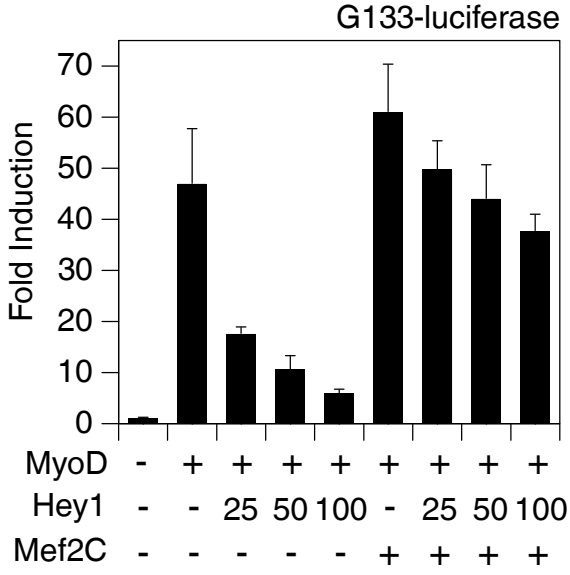

Figure 3.8. Forced Mef2C expression partially rescues MyoD-mediated induction of the Myogenin promoter in the presence of Hey1. 10T1/2 cells were transfected with 25 ng pRL-TK-Renilla, 25 ng G133-luciferase, 25 ng of pEMSV-MyoD, 25-100 ng pcDNA3.1-Hey1-V5, and 100 ng pcDNA3.1-Mef2C ($\alpha 1\beta$), as indicated. Firefly luciferase values were normalized to Renilla luciferase and plotted as averages of three replicate samples +/- standard deviation.

(E1, CAGTTG), with an additional E-box (E2, CACATG) and N-box (N1, CACCAG) located within the 400bp proximal to the start site. The Mef2C minimum promoter contains a single E-box (2C, CAGGTG). I performed EMSAs using in vitro transcribed and translated (TNT) Hey1-V5 and a labeled probe containing the Hey1 consensus E-box (HCE). The Hey1 complex ran with the same mobility as a complex present in TNT lysates, so I evaluated Hey1 binding after shifting the complex to a slower mobility with an anti-V5 antibody (Figure 3.9, lanes 1-3). As expected, addition of cold HCE competitor, but not mutant HCE, completely eliminated the binding of Hey1 to labeled probe (lanes 4-5). Addition of competitor DNA containing the various E-boxes and N-box found in the Myogenin and Mef2C promoters only marginally reduced formation of the Hey1 complex, despite a 50X molar excess of cold DNA (Figure 3.9, lanes 6-9). Other reports have also failed to show association of Hey1 with the high-affinity MyoD E-box (CAGGTG) (Fischer et al., 2002; Pichon et al., 2004). These data argue against robust DNA binding by Hey1 to known control elements within these myogenic promoters. Consistent with this conclusion, when I mutated the E1 site within the Myogenin promoter and carried out reporter assays in 10T1/2 cells, Hey1 still repressed MyoD-induced activity, indicating that inhibition is independent of this particular E-box (Figure 3.10). Efforts to map Hey1-responsive elements in the Myogenin promoter distinct from those necessary for induced activity were unsuccessful (data not shown).

Evidence for association of Hey1 with the Myogenin and Mef2C promoters in vivo

Despite the above findings in vitro, I sought to determine if Hey1 associates with the Myogenin and Mef2C promoters in vivo. Chromatin immunoprecipitation (ChIP)

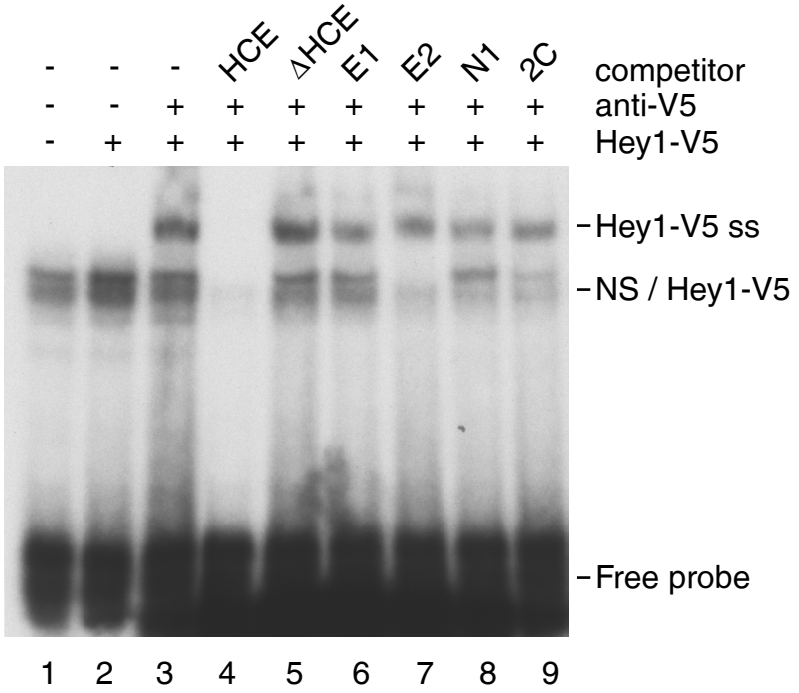

Figure 3.9. Evaluation of in-vitro DNA binding of Hey1 to myogenic promoter elements. TNT lysates programmed with either pcDNA3.1-V5/HisA empty vector or pcDNA3.1-TOPO-Hey1-V5 were incubated with a 22-nucleotide ^{32}P-labeled probe containing the Hey1 consensus target E-box (CACGTG). Anti-V5 antibodies and cold competitor probes (50X excess relative to labeled probe) were added as indicated prior to resolution of complexes by non-denaturing SDS-PAGE. HCE, Hey1-consensus E-box; ΔHCE, mutant Hey1-consensus E-box; E1, E2, E-boxes within the Myogenin proximal promoter; N1, N-box ~400bp upstream of Myogenin start site; 2C, high-affinity MyoD E-box within the Mef2C proximal promoter.

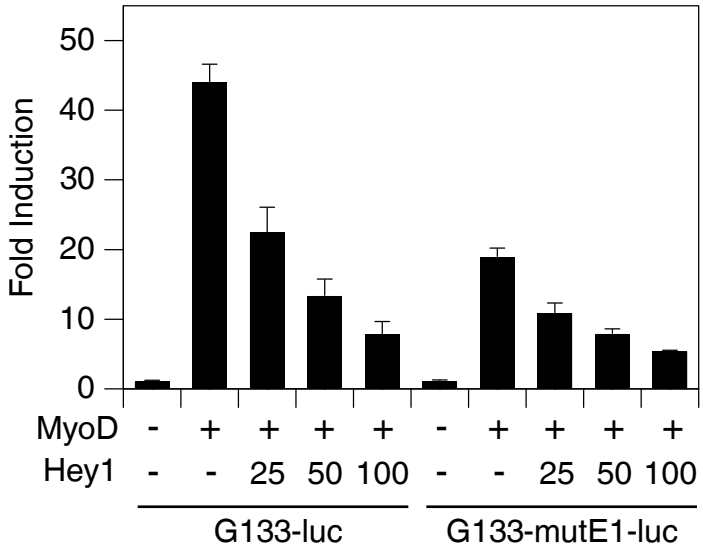

Figure 3.10. Hey1 represses induction of a Myogenin minimal promoter with a mutated E-box DNA element. 10T1/2 cells were transfected with 25 ng pRL-TK-Renilla, 25 ng G133-luciferase or G133-mutE1-luciferase, 25 ng of pEMSV-MyoD, and 25-100 ng pcDNA3.1-Hey1-V5. Firefly luciferase values were normalized to Renilla luciferase and plotted as averages of three replicate samples +/- standard deviation.

assays yielded inconsistent and variable results; accordingly, I turned to DamID, an assay that is better suited for detecting potentially weak or indirect interactions with DNA in vivo (van Steensel and Henikoff, 2000, 2003). In this assay, a protein of interest is fused with the bacterial DNA adenine methyltransferase (Dam) and then expressed at low levels in mammalian cells. If the chimeric protein associates with particular regions of DNA, then only the targeted DNA becomes methylated since mammalian cells lack Dam. Methylated regions are revealed on the basis of cutting by the methylation-specific restriction enzyme DpnI and subsequent amplification of digested fragments (Reddy et al., 2008; Vogel et al., 2007). This assay is capable of detecting even transient and weak interactions between the chimeric proteins and their target DNA.

I generated lentiviruses that express MyoD-Dam and Hey1-Dam. Expression and activity of the fusion proteins were confirmed with Western immunoblots and reporter assays, respectively (Figure 3.11). When transiently transfected, the pLgw-based lentiviral vectors employ a strong CMV promoter to drive high-level expression. This promoter is deleted following infection and integration, and subsequent low-level expression from the virus DNA is controlled by an un-induced heat shock promoter. I infected C2C12 cells with individual lentiviruses, and genomic DNA was isolated and processed (see Materials and Methods). Amplified (i.e. methylated) DNA was interrogated for genes of interest using quantitative PCR. Cells infected with a Dam-only virus were used to control for background methylation. As expected, cells infected with the MyoD-Dam virus gave rise to a ~3 fold relative increase in methylation in the vicinity of the Myogenin and Mef2C promoters, but not in the vicinity of the GAPDH promoter or immunoglobulin heavy-chain (IgH) enhancer (Figure 3.12A). Importantly, cells

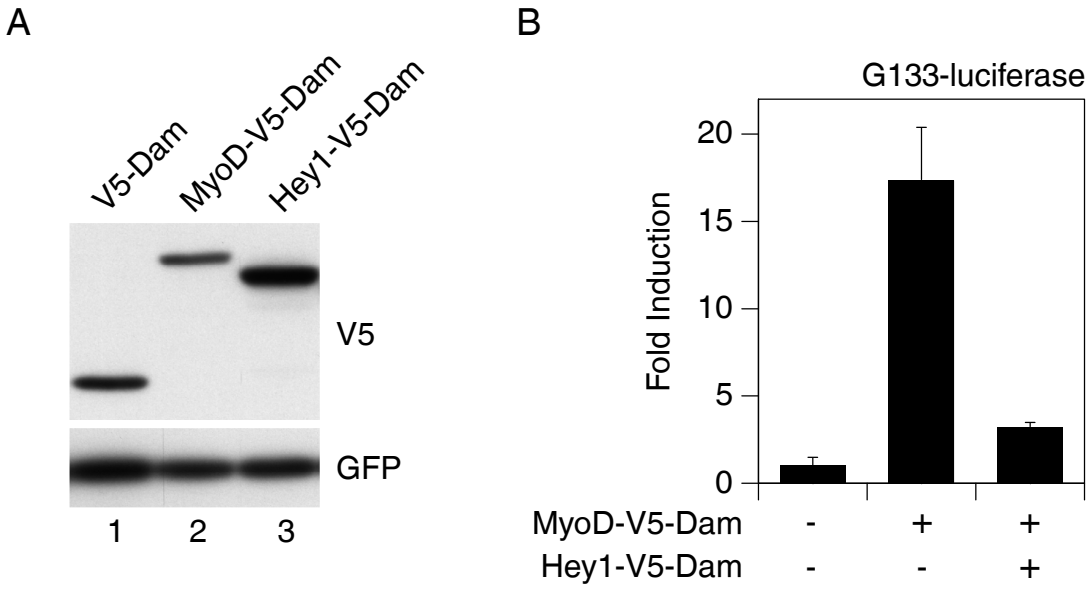

Figure 3.11. Evaluation of protein expression and activity of Dam fusion constructs. (A) 293T cells were transiently transfected with 2 µg of either pLgw-V5-EcoDam, pLgw-MyoD-V5-EcoDam, or pLgw-Hey1-V5-EcoDam and 2 µg CMV-GFP. Lysates were harvested after 48 hours and subjected to Western immunoblot analysis with anti-V5 or anti-GFP antibodies. (B) 10T1/2 cells were transfected with 25 ng pRL-TK-Renilla, 25 ng G133-luciferase, 25 ng of pLgw-MyoD-V5-EcoDam, and 50 ng pLgw-Hey1-V5-EcoDam, as indicated. Firefly luciferase values were normalized to Renilla luciferase and plotted as averages of three replicate samples +/- standard deviation.

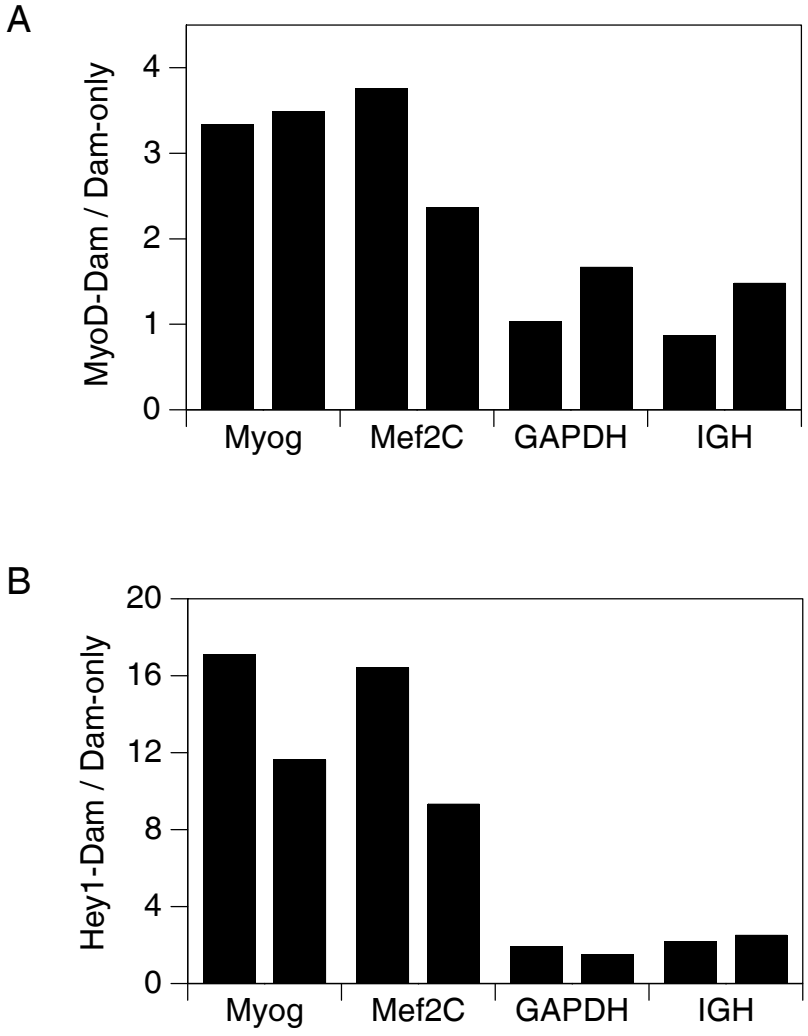

Figure 3.12. Hey1 associates with the promoter regions of endogenous target genes in cultured myoblasts. C2C12 cells were infected with pLgw-V5-EcoDam, pLgw-MyoD-V5-EcoDam, or pLgw-Hey1-V5-EcoDam lentiviruses, maintained for two days in growth medium (GM), and then switched to differentiation medium (DM) for 24 hours. Genomic DNA was harvested, subjected to the DamID protocol, and analyzed by quantitative PCR using primers in proximity to the promoter regions of Myogenin, Mef2C, and GAPDH, or within the immunoglobulin-heavy-chain enhancer (IgH). PCR values are presented as ratios of the MyoD-Dam (A) or Hey1-Dam (B) signal to the Dam-only signal. Data from two independent experiments are plotted.

infected with the Hey1-Dam virus also generated significantly increased relative methylation (~12 fold) in the vicinity of the Myogenin and Mef2C promoters (Figure 3.12B). These data indicate that Hey1 associates specifically with the Myogenin and Mef2C genes, and this likely leads to their transcriptional repression.

I next asked if targeting of Hey1 has consequences for the recruitment of MyoD. I transduced C2C12 cells with either a pBABE-puro or a pBABE-FLAG-Hey1 retrovirus and switched the cultures from growth medium to differentiation medium for 40 hours. I then used ChIP to evaluate the recruitment of MyoD to the Myogenin and Mef2C promoters in each group of cells. I observed a reduction in MyoD recruitment in cells transduced with FLAG-Hey1 relative to those harboring the parental virus (Figure 3.13). As expected, Pol II recruitment was also reduced at the Myogenin promoter (Pol II was not detected at the Mef2C promoter for unknown reasons). Only negligible recruitment of either factor was observed at the silent immunoglobulin heavy-chain enhancer, which was used to normalize the data. Reduced MyoD recruitment in vivo is not likely to result from the downregulation of MyoD expression by Hey1, as MyoD RNA levels were only marginally reduced (Figure 3.14), and MyoD:E47 heterodimers were still observed in Hey1-expressing C2C12 cells (Figure 3.5). I conclude that forced expression of Hey1 results in compromised recruitment of the master regulator MyoD to its target gene promoters.

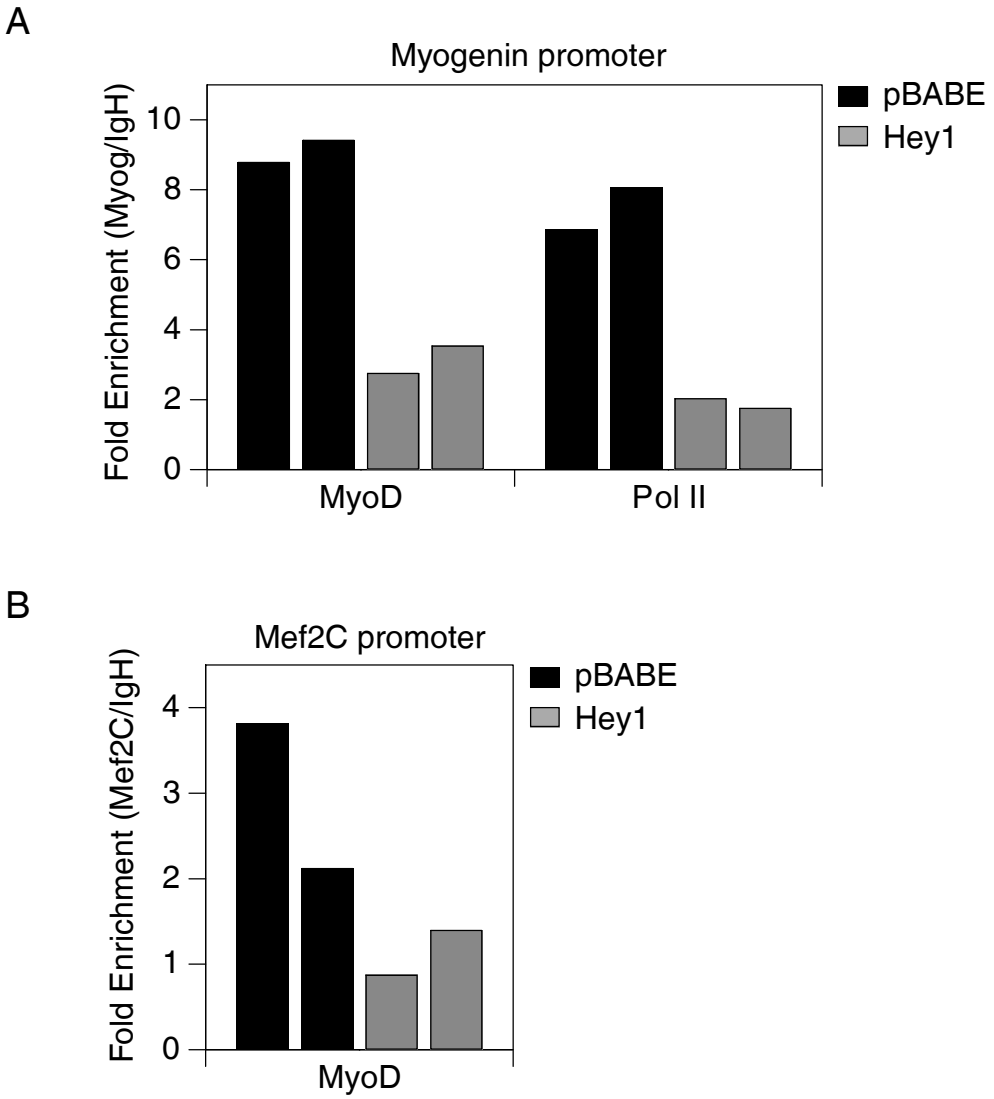

Figure 3.13. Constitutive expression of Hey1 in C2C12 myoblasts correlates with reduced recruitment of MyoD to target gene promoters. C2C12 cells stably transduced with either pBABE-puro or pBABE-FLAG-Hey1 retrovirus were switched to DM for 40 hours prior to fixation and harvesting for ChIP assays. Chromatin was immunoprecipitated with IgG or antibodies specific for MyoD or RNA Pol II. Samples were analyzed by quantitative PCR using primers specific for the Myogenin promoter (A) or Mef2C promoter (B) and IgH enhancer. Anti-MyoD to IgG or anti-Pol II to IgG ratios at the Myogenin and Mef2C promoters were normalized to those at the IgH enhancer. Data from two independent experiments are plotted.

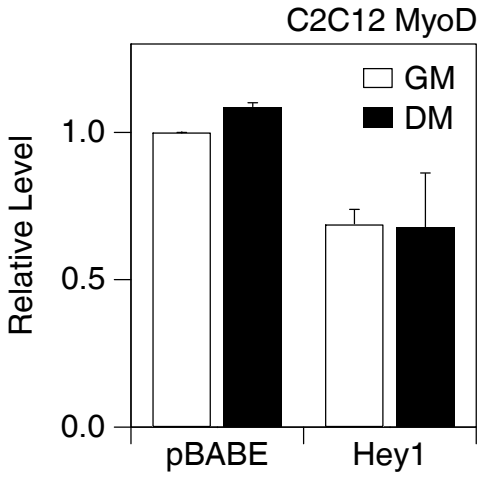

Figure 3.14. Determination of MyoD RNA levels in Hey1-expressing cells. C2C12 cells stably transduced with pBABE-puro or pBABE-FLAG-Hey1 retrovirus were maintained in growth medium (GM) or switched to differentiation medium (DM) for 24 hours. RNA was isolated and MyoD RNA levels were determined by quantitative RT-PCR.

DISCUSSION

Hey1 is one of several transcription factors downstream of Notch activation which may contribute to the pathway's inhibitory effects in muscle. In this portion of my thesis, I sought to determine the mechanism by which this canonical Notch effector mediates transcriptional repression. My studies on Hey1 support a model in which this bHLH inhibitor primarily functions by binding in the vicinity of the Myogenin and Mef2C promoters to shut off target gene expression. I present three independent and complementary lines of evidence that are consistent with this model and argue against a proposal that Hey1 sequesters MyoD into inactive heterodimers (Sun et al., 2001). First, MyoD-mediated induction of a direct MyoD target gene (Cadherin-15) or of an E-box-driven reporter (4RE-tk-luc) was resistant to repression by Hey1. Second, MyoD did not form heterodimers with Hey1 under the conditions of my co-immunoprecipitation assays, yet readily formed heterodimers with E47 as expected. Importantly, Hey1 did form homodimers. Third, MyoD:E47 heterodimers were unaffected in cells expressing Hey1, despite the inability of these cells to differentiate. While the basis for the discrepancy with a previous study (Sun et al., 2001) is currently unknown, my combination of functional and biochemical data strongly argue that Hey1 does not repress the intrinsic ability of MyoD to activate transcription.

Since Hey1 repressed the induction of Myogenin but not Cadherin-15, I sought to determine the basis for this promoter specificity. Past work had implicated the Mef2 family of transcription factors as critical mediators of Myogenin induction, in particular Mef2C (Dodou et al., 2003; Edmondson et al., 1992; Wang et al., 2001). While Hey1 did

not inhibit Mef2C activity, it did repress Mef2C expression, suggesting that repression of Myogenin might be due to the lack of Mef2C protein. However, forced expression of Mef2C only partially restored Myogenin promoter activity in the presence of Hey1, suggesting additional mechanisms. Indeed, DamID assays showed that Hey1 associates with both the Mef2C and Myogenin promoter regions, indicating that its ability to inhibit myogenesis may be due to the repression of multiple myogenic loci.

The work presented here raises several additional mechanistic questions of interest. If Hey1 associates with DNA in proximity to the Myogenin and Mef2C transcriptional start sites, where exactly does this binding occur, through what intermediates if any, and how does Hey1's presence translate into transcriptional silencing? The resolution of DamID has been reported to be ~1 kb at best (Greil et al., 2006) and thus cannot be used to identify specific binding sites occupied by Hey1 within these target promoters. My EMSA data argue that Hey1 is not likely to bind directly to E-boxes or N-boxes at these loci, leaving open the possibility that Hey1 may associate instead through distinct DNA-bound regulatory proteins. Aside from MyoD and Mef2, additional transcription factors such as Pbx1/Meis, MSY-3, Six1/4, and Ski also participate in the complex transcriptional control of the Myogenin locus (Berghella et al., 2008; Berkes et al., 2004; Spitz et al., 1998; Zhang and Stavnezer, 2009). Hypothetically, Hey1 could interact with any of these or other factors present at the Myogenin promoter. An intriguing possibility is that Hey1 functions in conjunction with GATA proteins, specifically GATA3. As noted previously, GATA3 is induced by Notch in C2C12 cells and inhibits differentiation when constitutively expressed. Past work in other systems has demonstrated physical interactions between Hey1 and GATA proteins (Elagib et al.,

2004; Fischer et al., 2005; Kathiriya et al., 2004). If GATA3 is found to associate with GATA binding sites in the Myogenin and/or Mef2C promoters, this factor could potentially serve as an adaptor protein for Hey1 recruitment.

Hey1's presence at these loci appears linked to a reduction in MyoD recruitment, but how this occurs remains to be determined. Importantly, expression of Hey1 in C2C12 cells did not significantly reduce expression of MyoD or its inherent ability to bind a target E-box in-vitro. Under one scenario, Hey1 could alter local chromatin structure by recruiting histone modifying enzymes such as HDACs, and thereby impede MyoD recruitment. Hey proteins have been shown to bind HDACs in vitro (Iso et al., 2001b), but many studies have indicated Hey-mediated repression is insensitive to trichostatin A (Elagib et al., 2004; Fischer et al., 2005; Garg et al., 2005; Nakagawa et al., 2000), calling into question the relevance of such associations. Furthermore, I found that Hey1 failed to significantly repress a reporter construct in which the 133-bp Myogenin proximal promoter fragment was fused to a strong basal thymidine kinase promoter (data not shown). Repression of the nearby TK promoter would be expected if Hey1 were binding to this DNA and recruiting HDACs. Under a different scenario, by associating with myogenic promoters, Hey1 might sterically occlude access of MyoD to its target E-box or interfere with its binding to Pbx1/Meis, homeodomain proteins postulated to facilitate initial (weak) recruitment of MyoD to the Myogenin locus (Berkes et al., 2004).

While these questions require additional investigation, my data provide strong evidence that Hey1 functions by physically associating with the promoter regions of two critical myogenic genes, Myogenin and Mef2C. Previous reports have often proposed that Hey family proteins silence transcription via protein-protein interactions with

transcriptional activators, but seldom demonstrated association with target gene promoters (Fischer and Gessler, 2007). My results using the sensitive DamID technique imply that such protein-protein interactions may often serve to tether Hey proteins to promoter regions rather than simply to sequester transcription factors away from DNA. Given that Mef2C is required for Myogenin induction, I initially explored the possibility that the Mef2C promoter alone may be the primary target of Hey1-mediated repression. Instead, my results indicate that Hey1 acts on both promoters, perhaps as a means to reinforce or lock in the repressed state through redundant mechanisms. It remains to be seen whether Hey1 also directly targets a much broader set of myogenic promoters, or whether specific features of the Myogenin and Mef2C loci render them uniquely receptive to Hey1 recruitment.

Chapter IV. Perspective and Future Directions

In total, my work has revealed an unanticipated level of complexity in the molecular regulation of skeletal myogenesis by the Notch signaling pathway. Contrary to past proposals that Notch functions simply by inducing the expression of the canonical targets Hes1 or Hey1, my results suggest that multiple effectors downstream of Notch activation likely contribute to myogenic repression. Some of these, like Hey1, appear to target specific critical promoters rather than silencing generalized MyoD transcriptional activity. The apparent redundancy of multiple mediators and the potential diversity of inhibitory mechanisms underscore the critical role that the Notch pathway plays in the developmental and regenerative biology of skeletal muscle.

When considered in the context of the Notch transcriptional network in muscle, my results on Hey1 suggest that individual Notch effectors may function through distinct yet complementary mechanisms to shut down myogenic gene expression. In contrast to Hey1, which appears to exert promoter-specific repression of myogenesis via association with key target loci, past studies on MyoR and Id3 have demonstrated that these factors directly repress the inherent activity of MyoD, the master regulator. As mentioned previously, Id3 likely acts by sequestering MyoD or E2A proteins into inactive heterodimers (Benezra et al., 1990; Jen et al., 1992). MyoR has similarly been reported to titrate E2A away from MyoD, but also directly binds E-box DNA elements in vitro (Lu et al., 1999). My own data on MyoR were largely consistent with these past findings (Figures 4.1-4.3). I found that MyoR dimerizes with E47 but not MyoD, binds to an E-box probe in vitro, and represses MyoD-mediated induction of both the Myogenin

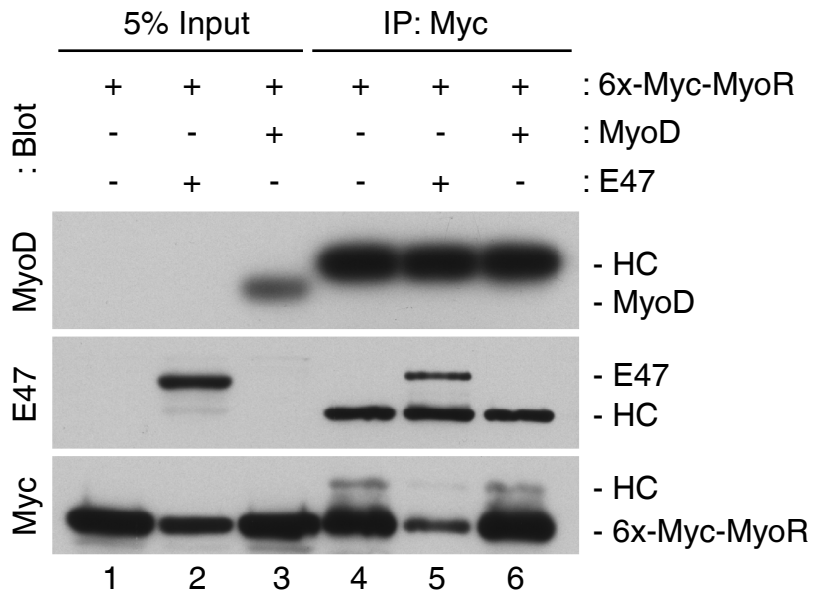

Figure 4.1. MyoR forms heterodimers with E47, but not with MyoD. 293T cells were transfected as indicated with 2 µg pcDNA-6X-Myc-MyoR alone or in combination with 2 µg CMV-E47 or 2 µg pEMSV-MyoD. Lysates were harvested after 48 hours and immunoprecipitated with Myc-specific antibodies. Immunoprecipitates and input samples were Western immunoblotted with anti-MyoD, anti-E47, or anti-Myc antibodies. HC, heavy chain.

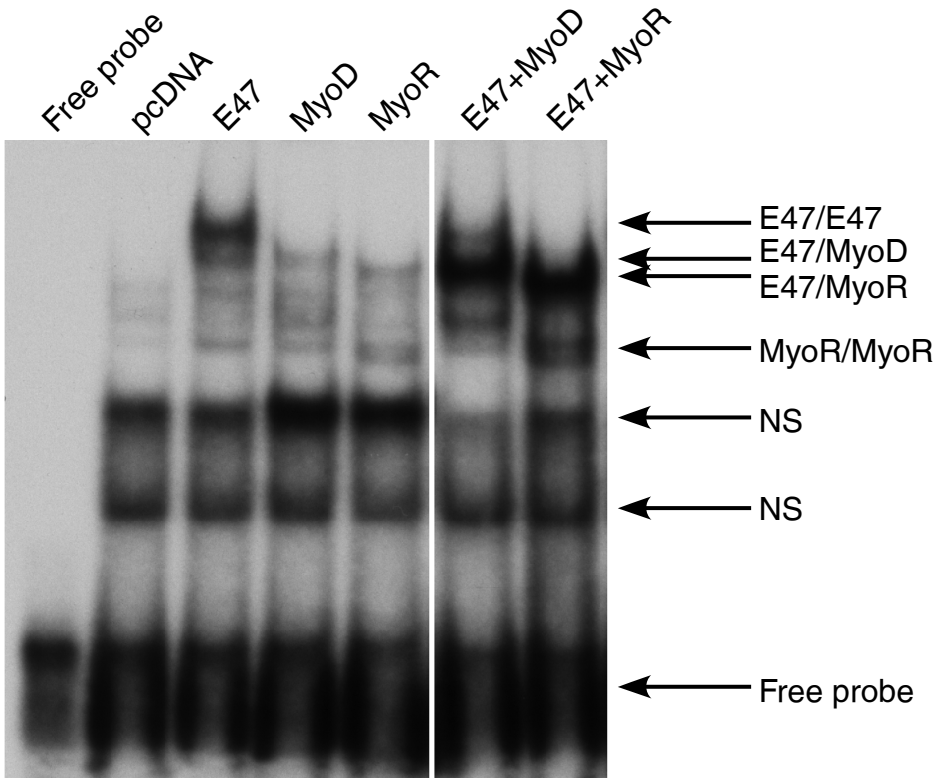

Figure 4.2. MyoR-E47 heterodimers and MyoR homodimers bind an E-box element in vitro. Nuclear extracts were harvested from 293T cells transiently transfected with either 6 μg pcDNA3.1 empty vector or 2 μg of individual plasmids as indicated (CMV-E47, pEMSV-MyoD, pcDNA-6X-Myc-MyoR). Total transfected DNA was kept constant (6 μg) by adding pcDNA3.1 empty vector as needed. Extracts were incubated with a 30-nucleotide ^{32}P-labeled probe containing the high-affinity MyoD E-box (CAGGTG) found within the Mef2C promoter and MCK enhancer. Protein-DNA complexes were resolved via non-denaturing SDS-PAGE. Complexes were identified (preliminarily) by expected altered mobility patterns relative to the E47 homodimer.

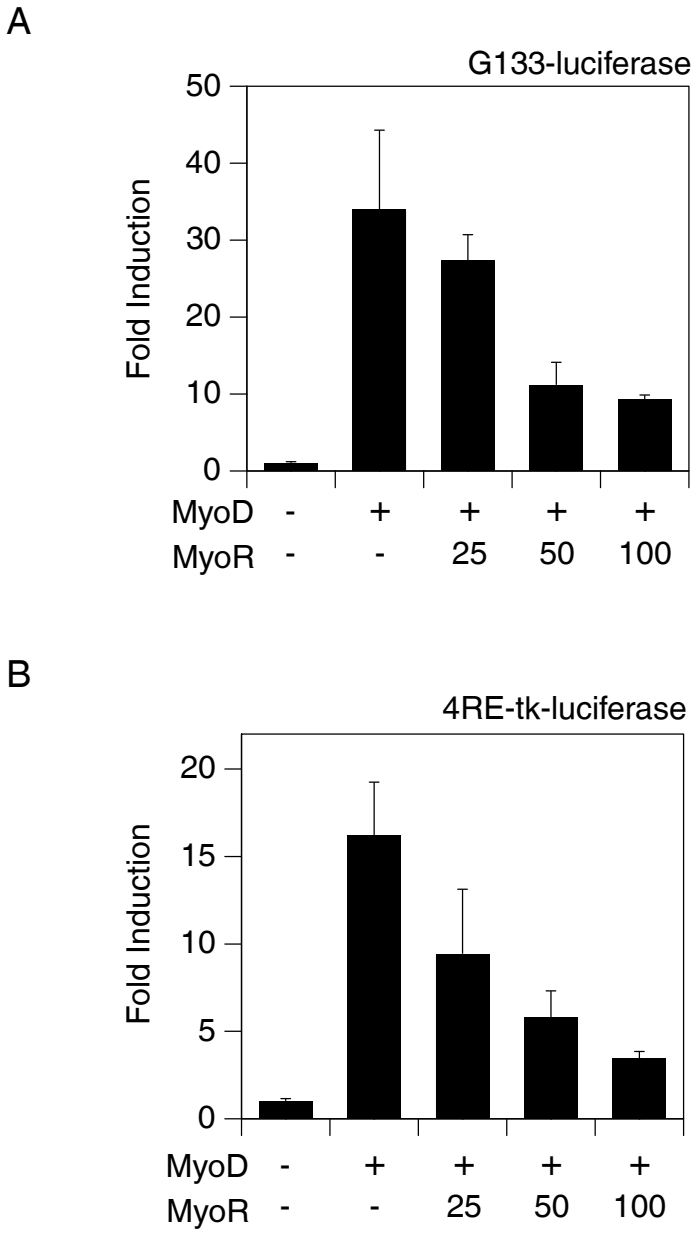

Figure 4.3. MyoR inhibits MyoD-mediated activation of a Myogenin reporter and a pure E-box-driven reporter. 10T1/2 cells were transfected with 25 ng pRL-TK-Renilla, 25 ng G133-luciferase (A) or 25 ng 4RE-tk-luciferase (B), 25 ng pEMSV-MyoD, and 25-100 ng of pcDNA3.1-MyoR-V5. Firefly luciferase values were normalized to Renilla luciferase and plotted as averages of three replicate samples +/- standard deviation.

promoter and a pure E-box-driven reporter. Whether ligand-induced levels of MyoR and Id3 are sufficient to bring about these effects on MyoD remains an open question. Interestingly, formation of endogenous MyoD:E47 complexes in C2C12 cells was not significantly affected by ligand stimulation (Figure 4.4). This finding suggests that endogenous levels of MyoR and Id3, if important for myogenic inhibition, may potentially act through mechanisms different from those described for the constitutively expressed proteins.

In light of the above results, one general limitation of my work relates to the use of constitutive overexpression in the functional analysis of Notch-responsive genes. While my studies revealed that at least four such genes (Hey1, MyoR, Id3, Gata3) can mimic the inhibitory effects of the pathway in muscle, my stably transduced cell lines expressed these genes at levels substantially higher than those observed following ligand-based stimulation. Just as past work has revealed that NICD overexpression can result in the activation of target genes not induced by Notch ligands (Iso et al., 2001a), so the use of overexpressed effectors could result in artifactual functional effects on differentiation. To address this concern, future studies will invoke an inducible system, in which expression levels of stably integrated transgenes can be finely tuned to more faithfully recapitulate ligand-mediated signaling. This approach will enable me to determine if expression of a given individual target at physiological levels is indeed sufficient to mimic Notch activation.

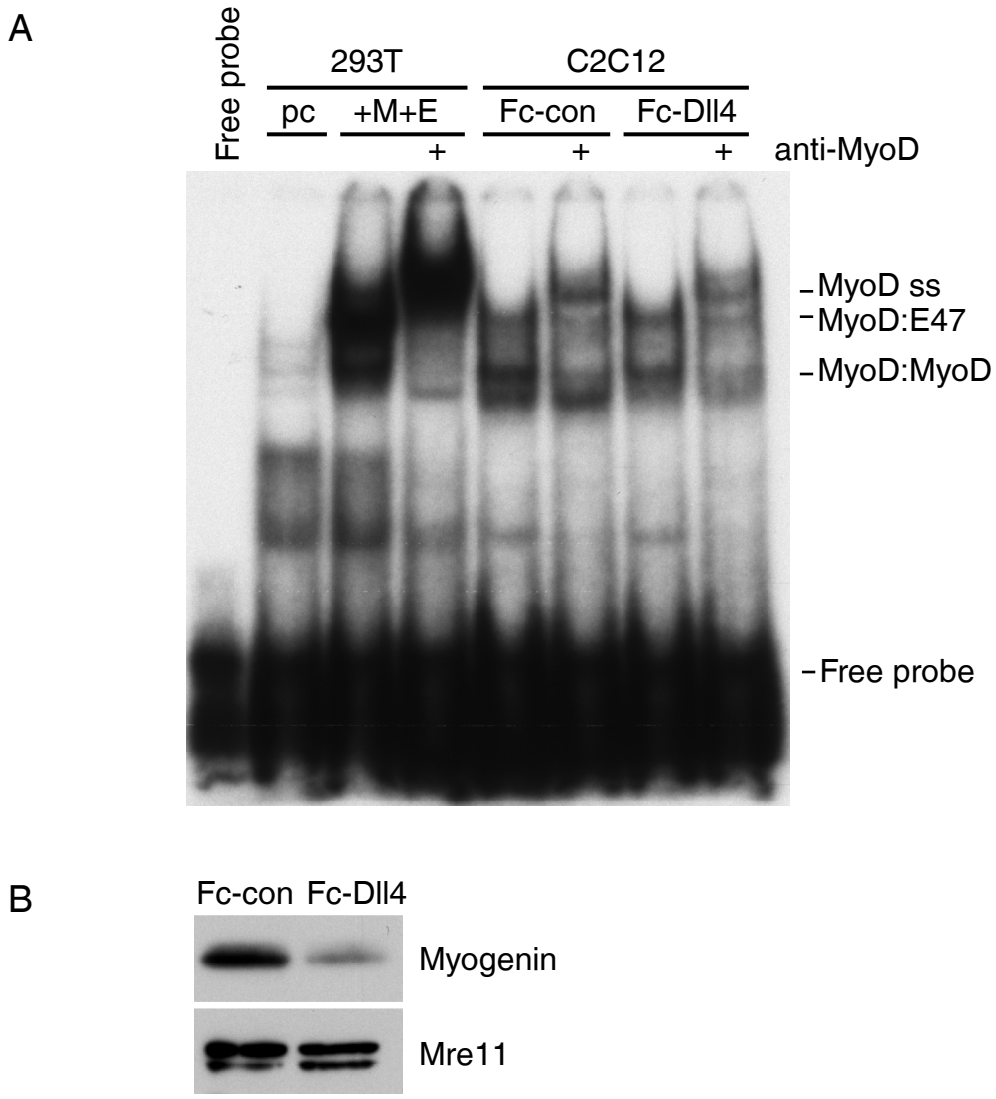

Figure 4.4. Fc-Dll4-mediated Notch signaling does not disrupt endogenous MyoD: E47 complexes in C2C12 cells. (A) Nuclear extracts were harvested from C2C12 cells seeded on plates coated with Fc-control or Fc-Dll4 ligands and maintained in differentiation medium (DM) for 24 hours. Nuclear extracts were also isolated from 293T cells transiently transfected with either 6 μg pcDNA3.1 empty vector or 2 μg pEMSV-MyoD, 2 μg CMV-E47, and 2 μg pcDNA3.1. Extracts were incubated with a 30-nucleotide ^{32}P-labeled probe containing the high-affinity MyoD E-box (CAGGTG) found within the Mef2C promoter and MCK enhancer. Protein-DNA complexes were incubated with or without MyoD-specific antibodies prior to resolution via non-denaturing SDS-PAGE. Supershifted MyoD-containing complexes are indicated "MyoD ss". (B) Nuclear extracts used in (A) were Western immunoblotted with antibodies specific for Myogenin or Mre11 as a loading control.

Functional studies in primary myoblast cultures and mice

A central future endeavor will be to extend my results in C2C12 cells to primary myoblast cultures and in vivo mouse models. While C2C12 cells exhibit many of the core properties of myoblasts in vivo, this cell line has undoubtedly undergone changes as a consequence of immortalization and extended culturing over three decades, and cannot be assumed to recapitulate all aspects of myoblast or satellite cell biology. Indeed, one important observation made by Rando and colleagues was that Notch activation in cultured primary myoblasts resulted in increased cellular proliferation (Conboy and Rando, 2002). Work from our own lab, by contrast, has failed to detect a significant effect of ligand-mediated stimulation on cell number or cell cycle exit in C2C12 cells; furthermore, forced cell cycle exit via p21 adenoviral infection failed to rescue the block to myogenesis (Shara Kabak, unpublished observations). If the proliferative effect of NICD reported by Rando can be validated with ligand-based Notch signaling in primary myoblasts, it would be interesting to revisit the possibility that Notch may function in part by promoting the cell cycle to inhibit differentiation.

Along these lines, an intriguing feature of my array list of Notch-induced genes was the presence of three growth factor receptors: epidermal growth factor receptor (EGFR), leukemia inhibitory factor receptor (LIFR), and platelet-derived growth factor receptor β (PDGFRβ). Furthermore, the cytoplasmic protein MIG6, a known inhibitor of EGFR (Zhang et al., 2007), was one of the few genes that was downregulated by ligand-mediated signaling. Induction of these cell-surface receptors by Notch (and repression of receptor inhibitors) might sensitize dividing satellite cells or myogenic progenitors to locally circulating growth cues and help maintain the proliferative, undifferentiated state.

In C2C12 cells, I validated EGFR and MIG6 as Notch-responsive genes by RT-PCR (Figure 4.5). I found that forced expression of EGFR is not sufficient to recapitulate the Notch-mediated block to differentiation observed in low serum medium (DM), although EGFR did sensitize cells to EGF-mediated inhibition (Figure 4.6). Given that immortalized C2C12 cells do not exhibit a proliferative response to Notch, a top priority will be to determine if constitutive expression of these growth factor receptors in primary myoblasts, preferably at levels induced by Notch ligands, phenocopies the effects of Notch activation; conversely, I will examine if siRNA knockdown of these receptors or their pharmacological inhibition impairs Notch-mediated responses. Interestingly, two studies in the past year have also reported EGFR as a target of Notch in different settings. Fine and colleagues demonstrated that Notch upregulates EGFR expression in human gliomas, potentially contributing to the pathway's oncogenic effects (Purow et al., 2008). Bray and colleagues, by contrast, showed that Notch directly induces EGFR in the Drosophila muscle cell line DmD8 (Krejci et al., 2009). Consistent with the growth factor model proposed above, further studies demonstrated that antagonism of EGFR signaling or loss of Notch function both result in premature differentiation of adult muscle progenitors in the fly (Krejci et al., 2009).

Beyond EGFR, the cytokine interleukin-6 (IL-6) is another validated Notch-responsive gene on my array list that deserves reexamination. While exposure of C2C12 cells to high levels of IL-6 had only modest inhibitory effects on differentiation (Figure 2.13), recent work has established a genetic requirement for IL-6 in satellite cell proliferation in vivo (Serrano et al., 2008). Interestingly, past reports have demonstrated that CSL associates with the IL-6 promoter in vitro (Kannabiran et al., 1997; Palmieri et

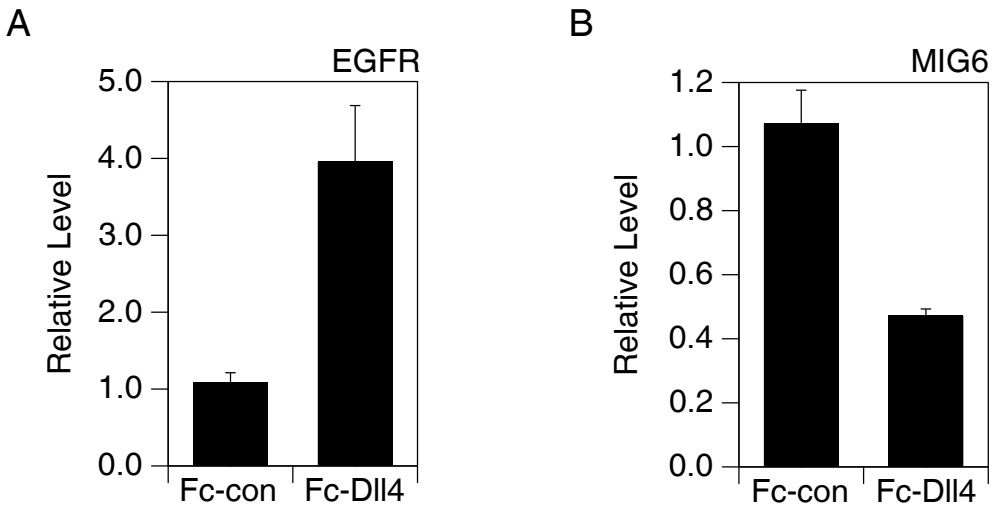

Figure 4.5. EGFR is induced and MIG6 is repressed by ligand-mediated Notch signaling in myoblasts. 10 cm dishes were coated with 2.5 ml of ligand-containing supernatant. C2C12 myoblasts were plated on either Fc-Dll4 or Fc-control ligand and propagated in growth medium (GM) for six hours. EGFR (A) and MIG6 (B) RNA levels were determined by quantitative RT-PCR using 18S as a loading control. Data represent averages +/- standard deviation of three replicate samples.

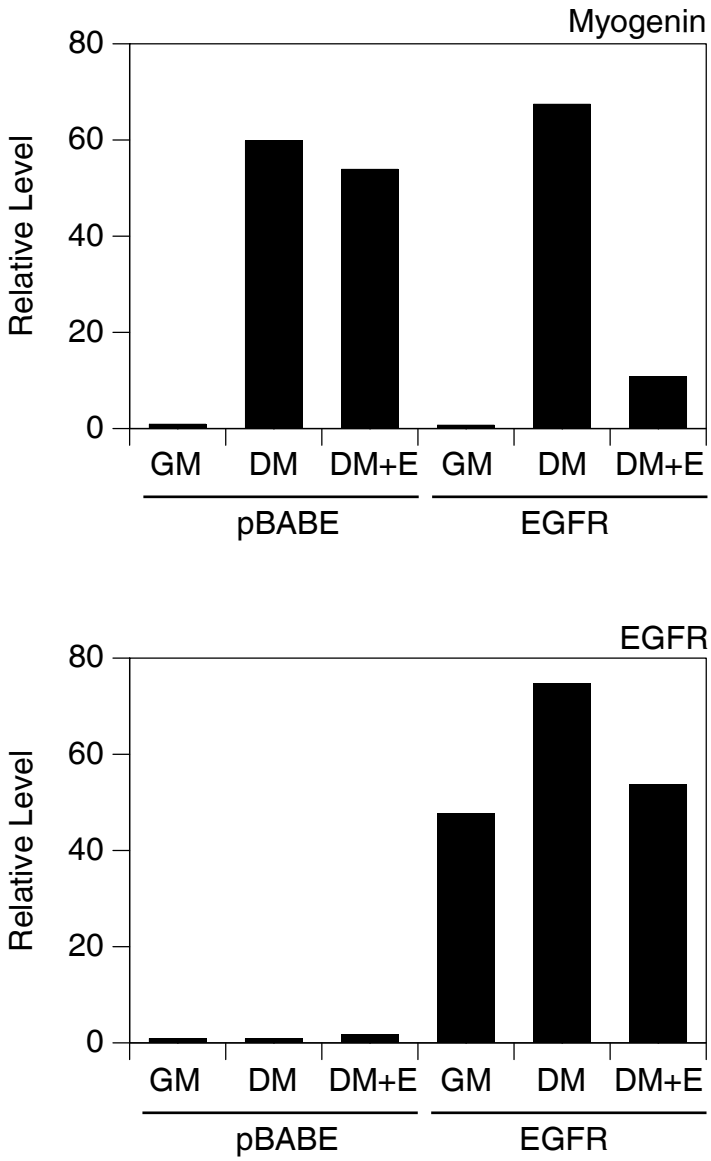

Figure 4.6. Constitutive expression of EGFR does not repress myogenesis, but sensitizes C2C12 cells to an EGF-mediated block to differentiation. C2C12 cells were stably transduced with parental retrovirus (pBABE-puro) or a retrovirus expressing EGFR. Lines were propagated in growth medium (GM) and then shifted to differentiation medium (DM) for 24 hours in the absence or presence (DME) of 100 ng/ml EGF. Myogenin (upper panel) and EGFR (lower panel) RNA expression levels were assessed by quantitative RT-PCR using 18S as a loading control.

al., 1999; Vales and Friedl, 2002), suggesting that this gene could be directly activated by Notch. It will be important to assess whether IL-6 levels increase upon muscle injury in a Notch-dependent fashion, as induction of this cytokine may represent yet another mechanism by which Notch could enhance the proliferative response of satellite cells. This could be tested by injecting a Notch inhibitor into injured muscle, as Rando and colleagues have described (Conboy et al., 2003), and examining local IL-6 production.

It remains to be determined whether the same Notch-responsive genes shown to repress myogenesis in our in vitro system also account for key functions of Notch during in vivo muscle development and regeneration. Single knockout animals for Hey1, MyoR, and Id3 exhibit no overt skeletal muscle defects (Fischer et al., 2004; Lu et al., 2002; Pan et al., 1999), suggesting that these effectors either play no role in myogenic development, or function redundantly with each other or additional factors. GATA3 null mice die during gestation (Pandolfi et al., 1995), necessitating the generation of a muscle-specific conditional mutant. One approach would be to cross the normal single knockouts above to generate double or triple knockout animals, and assess the formation of embryonic skeletal muscle. If these effectors are critical for Notch function, the expected phenotype would mirror that observed in the conditional CSL knockout or Dll1 null/hypomorph heterozygote—premature progenitor cell differentiation and ultimate muscle hypotrophy (Schuster-Gossler et al., 2007; Vasyutina et al., 2007). If the animals survive post-natally, muscle regenerative capacity could be evaluated following injury, with the expectation of compromised repair due to reduced satellite cell activation. Before initiating such studies, however, I would first opt for functional analyses of these and other Notch-responsive genes in primary myoblast cultures, as described above. If, for example, the EGF

receptor, alone or in combination with other effectors is shown to be critical for enhanced myoblast proliferation and impaired differentiation downstream of Notch activation, this gene and its partners would instead become the new focus for future in vivo explorations.

Expression profiling of the temporal response to Notch activation

While my array list of Notch-responsive genes in C2C12 myoblasts provided a solid starting point to probe the transcriptional output of the pathway in muscle, it remains possible that important targets were missed due to the selection of an early 6-hour time point. I chose this time point to bias the list of genes towards direct targets of the pathway and indeed captured several known CSL-dependent primary Notch effectors (Nrarp, Hey1, HeyL). To fully flesh out the broader scope of transcriptional changes downstream of ligand-mediated signaling, however, the gene profiling analysis should be extended to a time-course over 12-48 hours. Notch typically functions as a transcriptional cascade, in which primary targets like Hes/Hey proteins act as repressors to shut off expression of a set of secondary targets. However, in some cases, secondary or indirect targets may also be induced, perhaps as a consequence of "feed-forward" collaboration between NICD and one of its primary effectors. For example, in Th2 cells, Notch activates expression of GATA3, which then synergizes with NICD to induce the transcription of the cytokine IL-4 (Fang et al., 2007). In muscle, MyoR induction may reflect an analogous mode of regulation, as its expression is delayed relative to that of Hey1 and HeyL (Figure 4.7). By examining an extended temporal window following ligand stimulation, I would gain a clearer sense of secondary expression changes in muscle, whether positive or negative, which could offer important functional insights into

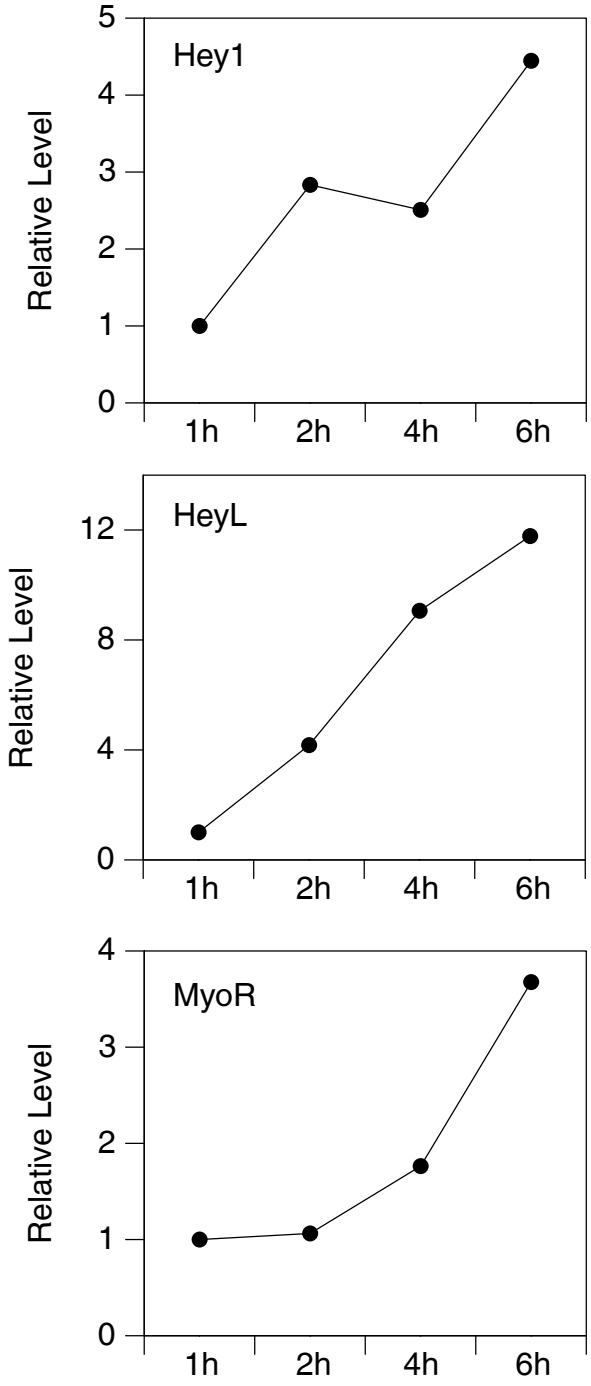

Figure 4.7. Induction of MyoR by ligand-mediated Notch signaling exhibits a temporal lag relative to that of Hey1 and HeyL. C2C12 cells were seeded on plates coated with Fc-Dll4 ligands, propagated in growth medium (GM) for one to six hours, and analyzed for expression of Hey1 (upper panel), HeyL (middle panel), and MyoR (lower panel) by quantitative RT-PCR using 18S as a loading control. Q-PCR values were normalized to the one hour time point, defined as "1".

the mechanisms of myogenic inhibition. My current list revealed only five genes, mostly not well characterized, whose expression declined after 6 hours, suggesting that this time point was too early to detect most secondarily repressed targets. Another interpretation, however, is that some genes targeted for repression by primary effectors may be silent by default in myoblasts, prior to differentiation, and hence would not be detected by my arrays (eg. Myogenin or Mef2C). One way to circumvent this problem would be to activate Notch signaling in cultures that had already initiated differentiation, and identify the specific transcripts that decline in abundance after a short duration (whether Notch activity can in fact reverse or impair the myogenic transcriptional program once initiated must first be verified).

Further functional analysis of Notch-responsive genes

One of the strategic questions I faced in my thesis project was how to functionally evaluate the list of genes obtained from my array experiment. The straightforward path that I chose was first to determine which individual genes out of a restricted subset were sufficient, when constitutively expressed in C2C12 cells, to phenocopy Notch-mediated repression of differentiation. Subsequently, I employed siRNAs against one or more genes capable of inhibition, in an attempt to define the set of effectors essential for the pathway's function. This approach had two main limitations, beyond the use of constitutive retroviral overexpression. First, cloning individual cDNAs into a retroviral construct and generating stable cell lines was a time- and labor-intensive process that severely limited the number of genes I could realistically test for function. Second, my

functional screening evaluated only single genes in parallel, without addressing potential combinatorial, collaborative actions of multiple targets.

In the future evaluation of this array data set or subsequent ones, I would consider alternative approaches. To address the above concerns, I would seek out higher-throughput strategies to functionally screen through the induced genes on my list. The cost of purchasing cDNA clones ready for use in expression plasmids has dropped significantly, which would facilitate the quick acquisition of a much larger pool of cDNAs. As a first pass, rather than relying on stable cell line creation, I would likely perform luciferase reporter assays in cultured myoblasts to screen for the ability of individual or multiple Notch-responsive genes to inhibit MyoD-mediated activation of a Myogenin reporter construct (G133-luciferase), a robust readout of the combined activities of MyoD and Mef2. Use of this readout should capture most potential inhibitors of the myogenic transcriptional program, as MyoD and Mef2 represent the central drivers of transcription in muscle. While results from this initial screen would rely on transient transfections and consequently on high levels of expressed gene products, I would then move to a system of stable inducible expression in C2C12 myoblasts. A subset of genes that exhibited the strongest inhibitory activity would be expressed under the control of a TET-inducible retroviral promoter, to dial down expression to ligand-induced levels. Ideally, this strategy would even allow for the assessment of potential synergistic or collaborative effects between a pair of effectors expressed off vector backbones with different selectable markers.

Examination of potential post-transcriptional effects of Notch

Notch activates a transcriptional cascade, and hence most of my efforts have been focused on identifying and investigating the function of genes upregulated in response to ligand-mediated signaling. Past work, however, has revealed that Notch signaling can also orchestrate the ubiquitin-mediated proteasomal degradation of various transcription factors, such as E2A and Tal1/SCL (Nie et al., 2008; Nie et al., 2003). Exactly how Notch facilitates the destruction of these proteins has not been elucidated, but such post-transcriptional effects may represent indirect consequences of Notch-mediated transcription. Indeed, reduced protein stability of E2A and Tal1/SCL was shown to depend on CSL function, suggesting that Notch may directly activate genes involved in the ubiquitination pathway. While transcription downstream of Notch likely remains the common denominator, these studies suggest that phenotypic consequences of Notch activity in some instances reflect alterations in protein stability. In future work, I would like to test the hypothesis that Notch signaling in muscle functions in part to promote (or inhibit) the protein turnover of selected targets. My own work has shown that the effect of Notch on E2A reported in lymphocytes appears to be tissue-specific, as ligand-mediated signaling in C2C12 myoblasts did not affect E47 protein levels (Figure 4.8). Nevertheless, other components of the myogenic program could potentially be targeted post-transcriptionally.

To determine if Notch alters the stability of cellular proteins in proliferating myoblasts, I would invoke a recently described high-throughput system devised by Elledge and colleagues, called global protein stability profiling (Yen et al., 2008). This approach relies on the use of fluorescence-activated cell sorting (FACS) and microarray

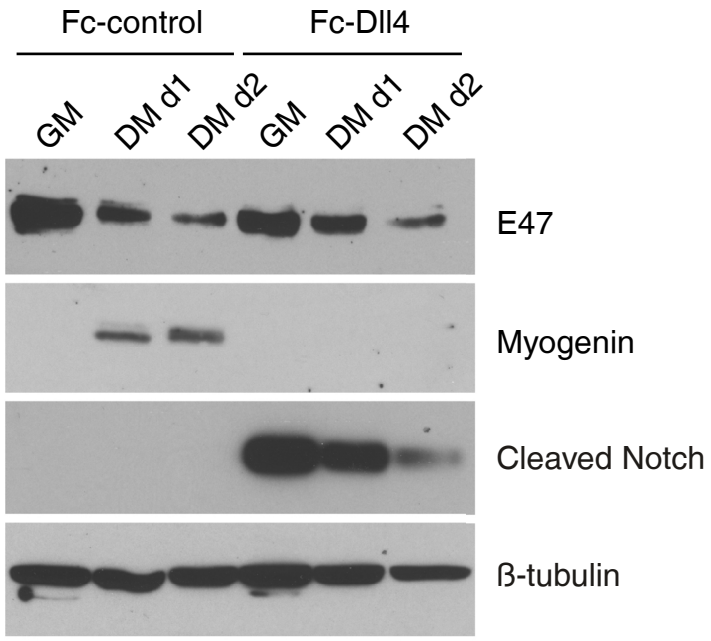

Figure 4.8. Ligand-mediated Notch signaling does not alter E47 protein abundance in proliferating or differentiating myoblasts. C2C12 cells were seeded on plates coated with either Fc-control or Fc-Dll4 ligands. Cells were propagated in growth medium (GM), shifted to differentiation medium (DM) for 1 or 2 days, and analyzed for expression of E47, Myogenin, and cleaved Notch1 by Western immunoblotting using ß-tubulin as a loading control.

analysis to infer the stability of a vast number of proteins simultaneously. In basic terms, the system employs a retroviral reporter construct that expresses a bicistronic message with an internal ribosome entry site (IRES). This message codes for two fluorescent proteins, a control protein DsRed, and a fusion protein between EGFP and a protein of interest (X). Since these proteins are expressed off the same RNA, they should be produced at the same ratio in transduced cells, and the EGFP/DsRed ratio reflects the stability of protein X. A cDNA library in this retroviral vector is employed to generate viruses and infect cells at low multiplicity of infection to make reporter cell collections. The cell library is fractionated by FACS into several pools based on increasing EGFP/DsRed ratios. Stability of a given protein "X" is inferred based on the distribution of cells expressing the protein X-EGFP fusion within the different pools (cells expressing a high-stability protein would be most concentrated in the pool with the highest ratio). High-throughput determinations are made possible by PCR-amplification of integrated virus-ORF identifier sequences from genomic DNA of sorted sub-pools and the total cell library; microarrays are then used to quantify the representation of each cDNA in a given sub-pool relative to the total cell population.

To apply this technology to my specific question, I would generate a C2C12 reporter cell library. One pool of library cells would be propagated on Fc-control ligands, and another on Fc-Dll4. Clone distribution profiles for each condition would be determined following cell library fractionation by FACS and microarray analysis. If a particular protein exhibited altered stability in the presence of an active Notch signal, this change would be reflected in a modified distribution of cells expressing that fusion protein in the fractionated pools. Assuming I am able to identify such targets, I would

first validate a subset of them by ascertaining the stability of the endogenous proteins via more traditional methods, such as pulse-chase experiments. Additional studies would be guided by several questions: Are these proteins functionally important in myogenesis? Is altered stability mediated through the ubiquitin-proteasome pathway? Does the effect depend on CSL-directed transcription?

Chapter V. Materials and Methods

Plasmids

Plasmids expressing the extracellular domains of Dll4 and Trail Receptor4 as Fc-fusion proteins were provided by Dr. Marion Dorsch (Millennium Pharmaceuticals, Cambridge, MA). Retroviral vectors encoding FLAG-tagged Hey1, Hey2, and HeyL were generated by sub-cloning the murine cDNAs, provided by Dr. Eric Olson (University of Texas), into the EcoRI site of the multiple cloning site of pBABE-puro-FLAG. Retroviral vectors for Nrarp, Trib2, and MyoR were generated by PCR amplification of the respective cDNAs from Notch ligand-stimulated C2C12 myoblasts followed by insertion into pBABE-puro at the BamHI/SalI sites (Nrarp) or the BamHI/EcoRI sites (Trib2, MyoR). The MyoR cDNA was also subcloned into the BamH1/EcoR1 sites of the expression vectors pcDNA-6X-Myc and pcDNA3.1-V5/HisA (Invitrogen, Carlsbad, CA). FLAG-tagged Nrarp and Trib2 were generated by PCR-subcloning the respective cDNAs into the SalI site (Nrarp) or EcoRI site (Trib2) of pBABE-puro-FLAG. G22Riken cDNA was obtained from Invitrogen (Carlsbad, CA) (clone 2649431) and sub-cloned by PCR into the BamHI/EcoRI sites of pBABE-puro. pBABE-HA-Id3 was generated by sub-cloning the HA-Id3 cDNA from RSV-HA-Id3 into the EcoRI site of pBABE-puro. The GATA3 retroviral vector was generated by PCR-subcloning the murine GATA3 cDNA, provided by Dr. Steve Reiner, into the EcoRI site of pBABE-puro-FLAG. The human EGFR cDNA, provided by Dr. Mark Lemmon, was excised from pcDNA3.1-EGFR using the PmeI and XhoI restriction sites and ligated into the SnaBI and SalI sites of pBABE-puro. MyoR reporter constructs were generated by PCR amplification of the indicated elements

from BAC RP23-398C14 (Invitrogen, Carlsbad, CA) followed by ligation into the KpnI/BglII sites of pGL3-basic (CR1), the KpnI/BglII sites of pGL2-promoter (CR2), or the KpnI site of pGL2-promoter (CR3). Three CSL sites present within CR3 were mutated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as follows: CSL site #1 was changed from "TTCCCACA" to "GGTACCCA"; CSL site #2 was changed from "TTCCCACG" to "GGTACCCG"; CSL site #3 was changed from "TTCCCA" to "TGTACA". CMV-NICD has been described previously (Ross and Kadesch, 2001).

G133-luciferase was provided by Vittorio Sartorelli (National Institutes of Health) and contains the 133-bp Myogenin proximal promoter fused to luciferase (Xu and Wu, 2000). pcDNA3.1-Mef2C (α1ß splice isoform) and 3x-Mef2-tk-luciferase were provided by Tod Gulick (Harvard Medical School). 3x-Mef2-tk-luciferase contains three copies of a Mef2 binding element fused to a minimal thymidine kinase (tk) promoter driving firefly luciferase (Zhu and Gulick, 2004). pEMSV-MyoD and 4RE-tk-luciferase were provided by Eric Olson (University of Texas). 4RE-tk-luciferase contains 4 copies of the MCK enhancer right E-box fused to a minimal tk promoter driving firefly luciferase (Lu et al., 2000). pcDNA3.1-TOPO-Hey1-V5 was generated by inserting the Hey1 cDNA (provided by Eric Olson) into the TOPO recognition site of pcDNA3.1D/V5-His-TOPO (Invitrogen, Carlsbad, CA). pcDNA3.1-Hey1-V5 was generated by PCR sub-cloning the Hey1 cDNA into the BamHI/EcoR1 sites of pcDNA3.1-V5/HisA (Invitrogen). G133-mutMef2-luciferase and G133-mutE1-luciferase were generated by QuikChange-mediated mutagenesis of the G133-luciferase Mef2 element (from [CTATATTTAT] to [CTATACTTTAT] (Edmondson et al., 1992)) or E1 element (from [CAGTTG] to

[AATTCG]), respectively. CMV-E47 has been described (Shen and Kadesch, 1995). DamID lentiviral vectors pLgw-RFC1-V5-EcoDam and pLgw-V5-EcoDam (Vogel et al., 2007) were provided by Bas Van Steensel (Netherlands Cancer Institute). pLgw-MyoD-V5-EcoDam and pLgw-Hey1-V5-EcoDam were generated via the Gateway recombination system (Invitrogen, Carlsbad, CA). The MyoD and Hey1 cDNAs were first sub-cloned by PCR into the Gateway entry vector pENTR-3C. Resulting entry clones were then recombined using LR Clonase II with the lentiviral destination vector pLgw-RFC1-V5-EcoDam. pVSVG, pGag/Pol, and pRSV-REV were provided by Carl June (University of Pennsylvania). All plasmids generated by PCR were verified by sequencing.

Cell culture

C2C12 myoblasts, C3H 10T1/2 fibroblasts, and 293T cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) supplemented with L-glutamine and penicillin-streptomycin (growth medium, GM). Human skeletal muscle myoblasts (SkMC and HSMM) were purchased from Cambrex (East Rutherford, NJ) and maintained as directed by the manufacturer. For differentiation of myoblasts, cells were grown to near confluence and then shifted to DMEM containing 0.5% FBS (differentiation medium, DM).

Notch signaling was induced by exposing the cells to immobilized ligand. Conditioned medium was first prepared from 293T cells transfected with plasmids coding for fusion proteins between Fcγ of human IgG and either the extracellular domain of Notch ligand Delta-like-4 (Fc-Dll4) or that of Trail Receptor 4 (Fc-control). Culture

plates were initially coated for 1 hour at room temperature with 10 μg/ml anti-Fc antibody (Jackson Immunoresearch, West Grove, PA). The anti-Fc PBS solution was then aspirated and replaced by filtered conditioned medium described above. Following 1 hour incubation, supernatant was aspirated and cells were plated.

Transfections and luciferase assays

10T1/2 cells were transfected according to the FuGENE 6 protocol (Roche Diagnostics, Indianapolis, IN). For quantitative RT-PCR experiments, cells were seeded at a density of $5x10^4$ cells per well in 6-well plates and transfected with a total of 1.5 μg of DNA per well (pcDNA3.1/V5-HisA empty vector was used to keep the total amount of DNA constant). Cultures were maintained in growth medium (GM) for 1 day post-transfection and then switched to differentiation medium (DM) for 24 hours prior to isolation of RNA. For luciferase assays, cells were seeded at a density of $1x10^4$ cells per well in 24-well plates and transfected with a total of 300 ng DNA. Cultures were maintained for 1 day in GM post-transfection and then switched to DM for 24 hours prior to harvesting of lysates. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Transfections were normalized to Renilla luciferase (pRL-TK; Promega).

Microarray expression screen

$2.5x10^6$ C2C12 cells were plated on 10-cm dishes coated with either Fc-Dll4 or Fc-control ligand (2.5 ml per dish) and grown in GM for 6 hours. RNA was harvested with the RNeasy Kit (Qiagen, Valencia, CA). Three replicates were included for each

condition. RNA was submitted to the University of Pennsylvania Microarray Core Facility for subsequent transcript profiling analysis on Affymetrix MOE430v2.0 GeneChip arrays. Raw data was processed at the Penn Bioinformatics Core Facility using Array Assist Lite (Stratagene, La Jolla, CA), Spotfire (Tibco, Somerville, MA), and Significance Analysis of Microarrays (Stanford University).

Retroviral infections

Infections were performed as previously described (Pear et al., 1993) with minor modifications. Briefly, retroviral supernatants were harvested from 293T cells two days following FuGENE6 (Roche Diagnostics, Indianapolis, IN)-mediated transfection with 8 μg of the indicated pBABE vector and 2 μg of gag/pol and env helper plasmids. Supernatants were filtered (0.4 μm) to remove non-adherent 293T cells prior to direct use or storage at –80ºC. 18-24 hours prior to infection, C2C12 cells were plated on 6-well plates at a density of ~$1x10^5$ cells/well. Each well was incubated for 4-6 hours with 1.5 ml viral supernatant supplemented with 8 μg/ml polybrene. 24-48 hours following infection and subsequent re-plating on 10-cm dishes, selection was initiated with 2 μg/ml puromycin and continued for 3-5 days to obtain stable lines.

siRNA knockdown

C2C12 cells were transfected with 100-150 nM of the indicated SMARTpool siRNA oligonucleotides purchased from Dharmacon (Lafayette, CO). Transfections were performed as specified by the manufacturer using the Dharmafect#3 reagent. Briefly, myoblasts were plated on 12-well dishes at a density of ~$1x10^4$ cells per well the day

prior to transfection. One day post-transfection, cells were trypsinized and re-plated on ligand-coated 12-well plates. Wells were coated with 400 µl of Fc-control supernatant and either 15 µl (Figures 2.7 & 2.8), 100 µl (Figures 2.17 & 2.18), or 80 µl (Figures 2.19-2.21) of Fc-Dll4 supernatant; the total ligand volume on Fc-Dll4-coated wells was kept constant (400 µl) by mixing Fc-control supernatant as required. Cultures were switched from GM to DM one day following re-plating and harvested for RNA after an additional 24-72 hours as indicated. For the double knockdown, a mixture of 110 nM Hey1 siRNA and 40 nM MyoR siRNA was employed.

Semi-quantitative and quantitative RT-PCR

Total RNA was isolated from C2C12 cultures using the RNeasy kit (Qiagen, Valencia, CA). 0.125 to 2 µg of RNA was used to generate cDNA with the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). For semi-quantitative RT-PCR, 5% of the cDNA was included in each PCR reaction. Products were run out on 1.5% agarose gels and visualized by ethidium bromide staining. For quantitative PCR, TaqMan gene expression assays were employed for MyoD, Myf-5, Myogenin, Mef2C, Myh3, Hey1, Hey2, HeyL, MyoR, IL-6, Id3, ABF-1, Cadherin-15, GATA3, EGFR, MIG6, and 18S as an endogenous control (Applied Biosystems). 1-4% of a given cDNA reaction, 10 µl of 2X Taq Universal Mastermix, and 1 µl of the indicated 20X TaqMan assay were included in a 20 µl reaction volume per well. All reactions were performed in triplicate. Results were analyzed using the SDS2.2 Software (Applied Biosystems). Primer sequences used for SQ-RT-PCR are as follows:

HPRT 5’-GTTGGATACAGGCCAGACTTTGTTG-3’ and

5’-TGGGGACGCAGCAACTGACATTTCT-3’;

Myogenin 5'-GCGGACTGAGCTCAGCTTAAG-3' and

5'- GCTGTCCACGATGGACGTAAG-3';

MEF2A, 5'-TTGGAATGAACAGTCGGAAAC-3' and 5'-

CTAGTCCCTGTGGAGGCAAG-3’;

MEF2C, 5'-GAGAAGCAGAAAGGCACTGG-3' and 5'-

ATCTCACAGTCGCACAGCAC-3’;

MEF2D, 5'-AGCTCTCTGGTCACTCCTTCC-3' and 5'-

GCCCTGGCTGAGTAAACTTG-3';

GAPDH, 5'-AACGGATTTGGTCGTATTGGG-3' and

5'-TGGAAGGATGGTGATGGGATTTC-3';

Hey1 5’- GAAGCGCCGACGAGACCGAATCAA-3’ and

5’-CAGGGCGTGCGCGTCAAAATAACC-3’;

Hey2 5'-CGACGTGGGGAGCGAGAACAAT-3’ and

5'-GGCAAGAGCATGGGCATCAAAGTA-3';

HeyL 5’- GGTCCCCACTGCCTTTGAGA-3’ and

5’- TAGCTGACTGCTCAGGGAAGGCAA-3’;

Nrarp 5’- TGGTGAAGCTGTTGGTCAAG-3’ and 5’-

GTAGTTGGCGGGAAGGTACA-3’;

IL-6 5’- CCGGAGAGGAGACTTCACAG-3’ and 5’-

GGAAATTGGGGTAGGAAGGA-3’;

Trib2 5’- GCAACATCAACCAAATCACG-3’ and 5’-

GCGTCTTCCAAACTCTCCAG-3’; 8430408G22Rik 5’-CTCCTGCCACCCTGACTG-

3’ and 5’- TGGGCTGTGACCTTGTCC-3’; MyoR (Figures 2.9 & 2.10) 5’-GCTACGAGGACAGCTATGTGC-3’ and

5’-AGGAGGGCAAACAACACTTG-3’;

MyoR (Figure 2.14) 5’-GGGAGGATGCAAGAGGAAG-3’ and

5’-CGTCCAGAGACCACGAATG-3’;

Id3 5’-GCCTCTTGGACGACATGAA-3’ and 5’-GGCGTTGAGTTCAGGGTAAG-3’.

Western immunoblot analysis

Protein lysates for Western blots were prepared from cultured cells using RIPA lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) or a modified lysis buffer (10 mM Tris pH 7.3, 150 mM NaCl, 1% NP-40) supplemented with freshly added protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), 10 mM sodium fluoride, and 400 μM sodium orthovanadate. Lysates were incubated on ice for 15 minutes and cleared by centrifugation. Protein concentrations were determined using the DC Assay (Bio-Rad, Hercules, CA). 25-50 μg of lysate was added to 2X or 6X SDS sample buffer and boiled for 5 minutes prior to analysis by SDS-PAGE. Proteins were transferred to nitrocellulose and blotted with the following antibodies at the indicated dilutions: 1:1000 ß-tubulin (Sigma T-5293, St. Louis, MO), 1:500 Myogenin (Santa Cruz M-225, Santa Cruz, CA), 1:20 MHC (MF20, Developmental Studies Hybridoma Bank), 1:2000 cleaved Notch1 (Cell Signaling 2421, Danvers, MA), 1:500 FLAG (Abcam ab6711-200, Cambridge, MA) or 1:1000 FLAG (Sigma M2), 1:500 MyoR (Santa Cruz M-20), 1:5000 anti-Mre11 (Novus NB 100-142G1, Littleton, CO), 1:500 anti-GFP (Santa Cruz sc-8334), 1:1000 anti-E47

(BD/Pharmingen G127-32, San Jose, CA). After incubation with 1:2000 dilutions of HRP-conjugated anti-rabbit, anti-mouse, or anti-goat secondary antibodies (Amersham, Piscataway, NJ), bands were visualized via the LumiLight or LumiLight-plus detection system (Roche, Indianapolis, IN).

Co-immunoprecipitation assays

For immunoprecipitations, 293T cells were harvested ~48 hours post-transfection by scraping into lysis buffer (20 mM Tris-HCl pH 8.0, 100 mM KCl, 0.1% NP-40, 5 mM $MgCl_2$, 10% glycerol) supplemented with freshly added protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), 10 mM sodium fluoride, and 400 μM sodium orthovanadate (Iso et al., 2001b). Lysates were incubated on ice for 15 minutes and cleared by centrifugation. Protein concentrations were determined using the DC Assay (Bio-Rad Laboratories, Hercules, CA). Lysates were pre-cleared in lysis buffer supplemented with 50 μl proteinA/G PLUS-agarose and 4 μg normal rabbit IgG (Santa Cruz Biotechnology sc-2027, Santa Cruz, CA) for 2 hours at 4°C. 500 μg of pre-cleared lysate was incubated with 2 μg of anti-MyoD (Novocastra 5.8A, Newcastle upon Tyne, United Kingdom), anti-E47 (BD/Pharmingen G127-32, San Jose, CA), or anti-Myc (Santa Cruz Biotechnology sc-40 9E10, Santa Cruz, CA) antibody and 15 μl of protein A/G PLUS-agarose at 4°C overnight. Immune complexes were washed 4X with lysis buffer, eluted in 2X SDS sample buffer, and boiled for 5 minutes prior to resolution by SDS-PAGE. Proteins were transferred to nitrocellulose and blotted with the following antibodies at the indicated dilutions: 1:5000 anti-V5 (Invitrogen, Carlsbad, CA), 1:1000 anti-E47 (BD/Pharmingen), 1:500 anti-MyoD (Novocastra 5.8A), 1:1000 anti-Myc

(Santa Cruz 9E10). After incubation with a 1:2000 dilution of HRP-conjugated anti-mouse secondary antibody (Amersham, Piscataway, NJ), bands were visualized via the LumiLight or LumiLight-plus detection system (Roche Diagnostics, Indianapolis, IN).

Electrophoretic mobility shift assays (EMSAs)

Nuclear extracts for EMSAs were prepared from 293T or C2C12 cells using the NXTRACT CelLytic NuCLEAR Extraction Kit (Sigma, St. Louis, MO). 293T cells were transiently transfected with either 2 µg EMSV-MyoD and 2 µg CMV-E47, or 4 µg pcDNA3.1/Myc-HisC (Invitrogen, Carlsbad, CA), 48 hours prior to harvesting of extracts. C2C12 cells seeded on Fc-control or Fc-Dll4 ligands or stably transduced with pBABE-puro or pBABE-FLAG-Hey1 were maintained in 0.5% serum for 24 hours prior to extract isolation. Hey1-V5 was transcribed and translated in-vitro using the TNT T7 Coupled Reticulocyte Lysate System (Promega, Madison, WI). ^{32}P-labeled oligonucleotide probes containing the Mef2C E-box (Wang et al., 2001) or the Hey1 consensus target E-box (Fischer et al., 2002; Pichon et al., 2004) were prepared by end-labeling annealed oligonucleotides with [γ-^{32}P] ATP using T4 polynucleotide kinase (New England Biolabs, Ipswich, MA). Labeled probes were purified through G-25 Quick Spin Sephadex Columns (Roche Diagnostics, Indianapolis, IN) as specified by the manufacturer. 8 µg of nuclear extract or 8 µl of TNT lysate was incubated for 15 minutes at room temperature with 100,000 cpm of probe in a 15 µl binding reaction consisting of 0.2-1.0 µg poly dI:dC, 10 mM Tris pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, and 5.4% glycerol. Prior to addition of probe, extract was pre-incubated in binding buffer at room temperature for ten minutes. Where indicated, 50X excess of cold competitor probe

was added to the reaction. For supershifts, 1 μg of anti-MyoD (Novocastra 5.8A, Newcastle upon Tyne, United Kingdom) or 1 μg of anti-V5 (Invitrogen) antibody was added to the sample and incubated for an additional 15 minutes. Binding reactions were run out on 5% non-denaturing polyacrylamide TBE Ready Gels (Bio-Rad Laboratories, Hercules, CA) in 0.5X TBE. Gels were dried and visualized by autoradiography. Oligonucleotide sequences used for the generation of labeled probes and cold competitors are as follows, with E-boxes or N-boxes underlined: MEF2C.F GAGTGACATGAACAGGTGCACCCTGGCCT; MEF2C.R AGGCCAGGGTGCACCTGTTCATGTCACTC; HCE.F TCCAATGGCACGTGCCACTGCC; HCE.R GGCAGTGGCACGTGCCATTGGA; ΔHCE.F TCCAATGGGCCGTACCACTGCC; ΔHCE.R GGCAGTGGTACGGCCCATTGGA; E1.F CACCCAGCAGTTGGTGTGAG; E1.R CTCACACCAACTGCTGGGTG; N1.F TGCCCTGTCCACCAGCTGCCTTG; N1.R CAAGGCAGCTGGTGGACAGGGCA; E2.F GAAGGGGAATCACATGTAATCCACTG; E2.R CAGTGGATTACATGTGATTCCCCTTC.

DamID

DamID assays were carried out essentially as described (Vogel et al., 2007), with minor modifications. Briefly, lentiviral supernatants were harvested from 10-cm dishes of 293T cells on three consecutive days, two days following FuGENE6 (Roche Diagnostics, Indianapolis, IN)-mediated transfection with 10 μg of the indicated pLgw lentiviral vector, 3.5 μg of pVSVG, 6.5 μg of pGag/Pol, and 2.5 μg of pRSV-REV. Supernatants

were filtered (0.4 μm) to remove non-adherent 293T cells prior to storage at –80ºC. ~18 hours prior to infection, C2C12 cells were plated on 6-well plates at a density of $1x10^5$ cells/well. Each well was incubated overnight with 1.5 ml viral supernatant, diluted ~2:1 in growth medium. After removal of virus, cultures were maintained for two days in GM and then switched to DM for an additional 24 hours. Genomic DNA was isolated with the DNeasy Tissue Kit (Qiagen, Valencia, CA). Following ethanol precipitation of gDNA, DpnI digestion, ligation of adaptors, DpnII digestion, and ligation-mediated PCR (11 cycles of amplification in the final stage of PCR), samples were purified with Qiagen columns and diluted 1:60 in buffer EB prior to Q-PCR analysis (Reddy et al., 2008). 8 μl of a diluted sample was mixed with 10 μl 2X Power-SYBR green mastermix (Applied Biosystems, Foster City, CA), 1 μl of 2 μM forward primer, and 1 μl of 2 μM reverse primer. PCR primers were first tested on genomic DNA via semi-quantitative PCR to verify amplification of a single product of the expected size. Q-PCR reactions were also subjected to dissociation curve analysis. Primer sequences are as follows: Myog.F GTGGACTGGCACAGGAGAAC; Myog.R GTGGACTTGGGACAAAGCAG; Mef2C.F GAGAAGCAGAAAGGCACTGG; Mef2C.R CATTTCCAGCTCACTCATCATC; IgH.F GTCATGTGGCAAGGCTATTTG; IgH.R TTTGCTCAGCCTGGACTTTC; GAPDH.F CTCACGTCCCAACTCTCCAC; GAPDH.R GGCCTCCTATAGTATCCCTCCTC.

Primers for GAPDH and IgH are located directly within the proximal promoter and enhancer, respectively. Primers for Mef2C and Myogenin are located ~200 bp and ~700 bp downstream of the transcriptional start sites, respectively, due to the unfavorable

distribution of DpnI sites within the promoter regions; DpnI-generated fragments larger than 2 kb are not efficiently amplified in the ligation-mediated PCR step.

Chromatin immunoprecipitation assays

chIP was performed as previously described (Caretti et al., 2004), with minor modifications. C2C12 cells stably transduced with pBABE-puro or pBABE-FLAG-Hey1 were seeded on 15-cm dishes at a density of 1.25-1.4 x 10^6 cells per plate, maintained in GM for 2 days, and then switched to DM for 40 hours. Cultures were fixed in 1% formaldehyde for 10 minutes at room temperature, incubated for 5 minutes in 0.125 M glycine, washed twice in cold PBS, and scraped into 3 ml PBS. Following brief centrifugation, cell pellets were resuspended in 500 µl cell lysis buffer (5 mM Pipes pH 8.0, 85 mM KCl, 0.5% NP-40) supplemented with 1 mM PMSF and protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), incubated on ice for 10 minutes, and dounced 15X to facilitate nuclei release. After a 5-minute centrifugation at 5000 rpm, nuclei were resuspended in 300 µl nuclei lysis buffer (50 mM Tris-Cl pH 8.1, 10 mM EDTA, 1% SDS, 1 mM PMSF, protease inhibitor cocktail) and incubated on ice for 10 minutes. Samples were sonicated in ice water using a Misonix 3000 sonicator for three 10-second intervals interrupted by 1 minute rests, followed by a 10-minute centrifugation at 14,000 rpm at 4°C. Supernatants were transferred to clean tubes, diluted 1:10 with dilution buffer (0.5% Trition X-100, 2 mM EDTA, 20 mM Tris-Cl pH 8.1, 150 mM NaCl), and pre-cleared with protein A/G agarose-ssDNA (Upstate Biotechnology, Billerica, MA) for 2 hours. 250 µg pre-cleared chromatin was incubated with 4 µg of normal rabbit IgG (Santa Cruz sc-2027, Santa Cruz, CA), 4 µg of anti-MyoD (Santa Cruz

M-318X), or 4 µg anti-RNA-Pol II (Santa Cruz H-224X) antibody overnight with rotation at 4°C. Immune complexes were collected with BSA-blocked protein A/G agarose-ssDNA for 2 hours. Beads were washed eight times as follows: 2X buffer 1 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.1, 150 mM NaCl), 2X buffer 2 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.1, 500 mM NaCl), 2X buffer 1, 1X buffer 3 (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris, pH 8.1), 1X TE. Washed beads were incubated twice in 150 µl elution buffer (1% SDS, 100 mM $NaHC0_3$) for 15 minutes at 65°C. Pooled eluates were treated with DNase-free RNase (Roche Diagnostics, Indianapolis, IN) and incubated at 65°C overnight to reverse crosslinks. Following proteinase K treatment, phenol:chloroform extraction, and ethanol precipitation, samples were analyzed by Q-PCR. 2 µl of a 50 µl sample was mixed with 10 µl 2X Power-SYBR green mastermix (Applied Biosystems, Foster City, CA), 6 µl water, 1 µl of 2 µM forward primer, and 1 µl of 2 µM reverse primer. PCR primer sequences for the Myogenin promoter and IgH enhancer have been published (Bergstrom et al., 2002; Mal and Harter, 2003). Primers for the Mef2C promoter are as follows: Mef2C.F2 GAGCAGTTCTGTGTTCTTTTGC; Mef2C.R2 ATCCCTCTGCACAAGTGTCTG.

Chapter VI. References

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